

Projecto de investigação no âmbito do estudo EpiReumaPt

(Project Research under EpiReumaPt study)

Processo de submissão à Comissão de Ética

(Ethics Committee Submission)

1. Identificação do Projecto (Project identification)

a. Título do Projecto (Name of the Project)

Influence of the mtDNA haplogroups in the progression of osteoarthritis in different geographic populations

b. Investigador Principal (Principal Investigator):

Nome/Name: Francisco Javier Blanco García

Instituição/ Departamento Institution/Department:

Complejo Hospitalario Universitario A Coruña-INIBIC. Rheumatology service

c. Co-Investigadores (Co-Investigators):

1. Nome (Name) and Instituição/ Departamento (Institution/Department): Ignacio Rego Pérez/Complejo Hospitalario Universitario A Coruña-INIBIC. Rheumatology service
2. Nome (Name) and Instituição/ Departamento (Institution/Department): Angel Soto Hermida/Complejo Hospitalario Universitario A Coruña-INIBIC. Rheumatology service
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5. **Nome (Name) and Instituição/ Departamento (Institution/Department):** María Eugenia Vázquez Mosquera/Complejo Hospitalario Universitario A Coruña-INIBIC. Rheumatology service
6. **Nome (Name) and Instituição/ Departamento (Institution/Department):** Estefanía Cortés Pereira/Complejo Hospitalario Universitario A Coruña-INIBIC. Rheumatology servisse
7. **Nome (Name) and Instituição/ Departamento (Institution/Department):** Helena canhão/Serviço de Reumatologia e Doenças ósseas metabólicas do Centro Hospitalar Lisboa Norte- Hospital de Santa Maria; Faculdade de Medicina da Universidade de Lisboa; EpiReumaPt
8. **Nome (Name) and Instituição/ Departamento (Institution/Department):** Jaime Branco/ Serviço de Reumatologia do Centro Hospitalar Lisboa Ocidental- Hospital Egas Moniz/ CEDOC, Faculdade de Ciências Médicas da Universidade Nova de Lisboa; EpiReumaPt

2. Instituições/locais onde se irá realizar o projeto

(Department(s), Laboratory(ies), Institution(s) where the study will be developed)

Clinical data and samples were collected by EpiReumaPt study from Portuguese Society of Rheumatology

The laboratorial part of the project will be performed in the research laboratory of the INIBIC (Instituto de Investigación Biomédica de A Coruña)

3. Sumário (Summary) (Max: 150 palavras/words)

Recent findings in the last years point to the involvement of the mitochondrial dysfunction in the pathogenesis of osteoarthritis. Moreover, a key role of mtDNA polymorphisms, specifically the mtDNA haplogroups, also emerged by means of their influence on the prevalence, severity and progression of this disease in populations from Spain, the UK and China. Since the two main functions of the mitochondria are ATP production and heat generation, it has been

proposed that these specific mtDNA polymorphisms (haplogroups) are the result of a process of climate adaptation that permitted humans to adapt to colder climates; these mtDNA variants are inherited through the maternal lineage and influence our health today. Therefore, the purpose of this project is to analyze the influence of the mtDNA haplogroups on the (radiographic) progression of osteoarthritis in different geographic populations

4. Estado de Arte (State of Art): (Max: 1500 palavras/words)

Osteoarthritis (OA) is the most common joint disease and is among the most frequent and symptomatic health problems for middle aged and older people. Worldwide estimates are that 9.6% of men and 18.0% of women aged over 60 years have symptomatic osteoarthritis. About 80% of those with OA have significant limitations of movement and 25% cannot perform their major activities of daily living. The knee, the hip, and the hand are the most frequently affected sites for which patients seek medical care.

OA is characterized by focal areas of damage to the articular cartilage, by changes in the subchondral and marginal bone, and by a reaction in the soft tissues (synovium, ligaments, capsule) in and around the joint. Several data sets have led to the conclusion that mitochondria contribute to OA **pathogenesis** [1]. A significant decrease in mitochondrial complex II and III activity in OA chondrocytes compared with normal chondrocytes has been demonstrated, and the mitochondrial mass was also shown to be increased in OA chondrocytes [2]. In addition, the apoptotic mitochondrial pathway has been implicated as one of the major cellular pathways of apoptosis in OA chondrocytes. The inhibition of complexes III and V of the mitochondrial respiratory chain (MRC) causes an increased inflammatory response, which could be particularly relevant to the production of prostaglandin E₂ (PGE₂) and reactive oxygen species (ROS) [3]. There is also a reduced expression of MnSOD₂ in OA chondrocytes [4].

Although the aetiology of OA is not completely understood, some **OA risk factors** have been identified, such as obesity, previous trauma, misalignment or deformity of the joint and certain gene polymorphisms. These (interrelated) risk factors clearly illustrate the multi-factorial nature of the disease. The diagnosis of OA is rather unspecific and joint pain and dysfunction in combination with radiological features of joint degeneration are still considered the hallmarks of osteoarthritis. However, many subjects have radiological osteoarthritic features without joint pain and disease progression. Moreover, some patients are in a mild

stage of disease for many years or even decades while others progress rapidly, within a period of one or two years after the first signs of disease.

Frustratingly, we are currently unable to predict which OA patients are at **risk for rapid progression**. The lack of appropriate **biomarkers** that can monitor disease progression obstructs development of new interventions. Though promising biomarkers have been proposed, many are still in a rather experimental phase and large scale testing in clinical cohorts is needed to show their power in monitoring and/or predicting disease progression. These markers can in general be distinguished in three types: imaging markers such as semi quantitative measures from x-rays or MRIs, genetic markers such as polymorphisms in a number of genes and molecular markers such as various proteins, glycosaminoglycans and breakdown products in urine or blood.

Genetic markers for diagnosis or prognosis were discovered in large cohort studies [5] and can contribute to 20-50% of OA in specific joint locations. Given the importance of **mtDNA** to cellular physiology, in particular for energy production, generation of reactive oxygen species and regulation of cell survival, there are a number of studies investigating the association between mtDNA haplogroups and multifactorial diseases and aging. In this regard our group has led investigations of the possible role of mtDNA haplotypes in OA during the last 7 years. Our major findings are:

1. Haplogroup J from mitochondrial cluster TJ is associated with a lower incidence of knee [6] and hip [7] OA in the Spanish population
2. Patients with knee OA that carry the mtDNA haplogroup J may have a less severe form of the disease [6]
3. Collagen type-II biomarkers such as Coll2-1, Coll1-1NO₂, C2C and CPII are significantly influenced by the mtDNA haplogroups. Carriers of the haplogroup H showed the highest serum levels, meanwhile carriers of haplogroup J showed lower levels [8]
4. OA patients with radiological K/L grades II and III (the typical randomized cohort in most clinical trials) that carry the haplogroup H showed significantly increased serum levels of most of the above described biomarkers [8]
5. Haplogroup T from mitochondrial cluster TJ is associated with a lower incidence of knee OA in patients from the UK [9]

6. OA patients carrying the mtDNA haplogroup T (belonging to the mitochondrial cluster TJ) in the progression subcohort of the Osteoarthritis Initiative (OAI) show slower radiographic progression and less cartilage loss over time than patients that carry the mtDNA haplogroup H [*in press Arthritis. Rheumatol*, 2014]

All these findings may be explained by the decreased free radical (ROS and RNS) production of chondrocytes with haplogroups from the mitochondrial cluster TJ (haplogroups J and T) probably caused by reduced coupling efficiency of the oxidative phosphorylation system (OXPHOS), that leads to a decreased ATP production but also a lower ROS generation.

Based on these findings, the general goal of this proposal is to replicate in the Portuguese population previous findings by which the mtDNA haplogroups influence the radiographic progression of osteoarthritis. Specifically, we will try to differentiate forms of knee OA and rates of progression by detailed evaluation of a set of mtDNA haplogroups and radiographic markers.

Some relevant papers in the last years related to the objectives of this project (in order of citation in the background section):

1. Lopez-Armada MJ, Carames B, Martin MA, Cillero-Pastor B, Lires-Dean M, Fuentes-Boquete I, Arenas J, **Blanco FJ**. Mitochondrial activity is modulated by TNFalpha and IL-1beta in normal human chondrocyte cells. *Osteoarthritis Cartilage*. 2006 Oct 14(10): 1011-22
2. Maneiro E, Martín MA, de Andres MC, López-Armada MJ, Fernández-Sueiro JL, del Hoyo P, Galdo F, Arenas J, **Blanco FJ**. Mitochondrial respiratory activity is altered in osteoarthritic human articular chondrocytes. *Arthritis Rheum*. 2003;48:700-8.
3. Cillero-Pastor B, Caramés B, Lires-Deán M, Lema B, Ruiz-Romero C, Fuentes I, Galdo F, **Blanco FJ**, López Armada MJ. Mitochondrial Dysfunction Activates Cyclooxygenase 2 Expression in Cultures Normal Human Chondrocytes. *Arthritis and Rheumatism*. 2008; 58(8): 2409-2419.
4. Ruiz-Romero C, Calamia V, Mateos J, Carreira V, Martínez-Gomariz M, Fernández M, **Blanco FJ**. "Mitochondrial Dysregulation of Osteoarthritic Human Articular. Chondrocytes Analyzed by Proteomics: A Decrease in Mitochondrial Superoxide Dismutase Points to a Redox Imbalance." *Mol Cell Proteomics*. 2009, 8: 172–189.
5. Rego I, Fernández-Moreno M and **Blanco FJ**. "Genetics in osteoarthritis". *Current Genomics*. 2008, 9: 542-547.

6. Rego I, Fernández-Moreno M, Fernandez-Lopez C, Arenas J, **Blanco FJ**. Mitochondrial DNA haplogroups: Role in prevalence and severity of knee osteoarthritis. *Arthritis and Rheumatism* 2008; 58(8):2387-2396.
7. Rego I, Fernández-Moreno M, Fernández-López C, Gómez-Reino J, González A, Arenas J, **Blanco FJ**. “The role of European mtDNA haplogroups in the prevalence of hip osteoarthritis in Galicia (Northern Spain)” *Ann Rheum Dis*. 2010, 69:210-13.
8. Rego-Pérez I, Fernández-Moreno M, Deberg M, Pértega S, Fernández-López C, Oreiro N, Henrotin Y, **Blanco FJ**. Mitochondrial DNA haplogroups modulate the serum levels of biomarkers in patients with osteoarthritis. *Ann Rheum Dis*. 2010;69:910-917
9. Soto-Hermida A, Fernández-Moreno M, Oreiro N, Fernandez-Lopez C, Rego-Pérez I; **Blanco FJ**. Mitochondrial DNA haplogroups and osteoarthritis in different geographic populations. *Mitochondrion*. 2014;15:18-23.

5. Objectivos (Aims): (Max: 300 palavras/words)

SPECIFIC AIMS:

- 1- To assign the mtDNA haplogroups in Portuguese OA patients with Caucasian ancestry.
- 2- To analyze the influence of the mtDNA haplogroups in the progression of knee OA in the EpiReumaPt cohort

6. Desenho do estudo (Study design) (Max: 300 palavras/words)

This study attempts to assign the mtDNA haplogroups in the follow-up cohort of EpiReumatPt (the CoreumaPt) in order to analyze their influence on the radiographic progression over time of knee OA. To carry out this approach, we will assign the different Caucasian haplogroups by means of a sequencing-based method (single base extension) in 986 knee OA patients from the CoreumapPt cohort. We will take into account different confounder (clinical) variables (collected in this cohort and described in the variables description section), such as gender, age at baseline, body mass index at baseline, functional activity, etc to perform a logistic regression model that permits us to assess the influence of mtDNA haplogroups on radiographic progression over time (more than 2 years follow-up).

7. População/amostra (explicitar se o estudo envolve menores):

(Population/sample: clarify children enrollment, if applicable)

EpiReumaPt (Portuguese Epidemiologic Study of Rheumatic Diseases) was the first epidemiologic population based Portuguese study that aimed to determine rheumatic diseases prevalence that started in September of 2011 and finished in December 2013. It recruited 10,661 random participants representative of the adult Portuguese population and all of them were invited to be part of a cohort. The CoReumaPt is the cohort constituted by the 10661 subjects from EpiReumaPt that are being followed through phone calls interviews in order to constitute a national registry that will capture socioeconomic data, lifestyle trends, comorbidities, life quality and treatment patterns of patients with rheumatic diseases. All patients signed informed consent. Moreover, this study is conducted in accordance with the regulations governing clinical trials such as the Declaration of Helsinki, as amended in Seul (2008), and was approved by the local Ethic Committees of the regional health administrations and Ethic Committee of Nova medical School. The mtDNA haplogroups study will include the 986 Knee OA patients from CoReumaPt study.

7.1. Critérios de inclusão (inclusion criteria)

The mtDNA Haplogroup study will include the EpiReumaPt's population with Knee OA. The EpiReumaPt inclusion and exclusion criteria are described elsewhere (Ramiro S, Canhão H, Branco JC. EpiReumaPt Protocol - Portuguese epidemiologic study of the rheumatic diseases. Acta Reumatol Port 2010; 35:384-390).

7.2. Critérios de exclusão (exclusion criteria)

Subjects from EpiReumaPt without OA diagnosis. The EpiReumaPt inclusion and exclusion criteria are described elsewhere (Ramiro S, Canhão H, Branco JC. EpiReumaPt Protocol - Portuguese epidemiologic study of the rheumatic diseases. Acta Reumatol Port 2010; 35:384-390).

8. Descrição Detalhada (Metodologia) / Detail Description**(Methodology):** (Max: 2500 palavras/words)**Deverá incluir: (including)**

- a) tipo de estudo (transversal, longitudinal)/ Type of study
- b) descrição das variáveis independentes (exposição) e como serão medidas (variables description and how they will be measured)
- c) potenciais confundidores e como serão avaliados (confounders and how will be assessed)
- d) outcomes e a sua avaliação (outcomes and how will be assessed)

This is a longitudinal study that attempts to analyze the influence of the mtDNA haplogroups on the radiographic progression of osteoarthritis in patients belonging to the EpiReumaPt cohort. For this purpose, we will have access to those clinical and radiographic variables collected for this study in order to evaluate the radiographic progression over time adjusting for confounder variables such as gender, age, body mass index (BMI), functional indexes, life style, health status, morbidity, therapy and sociodemographic, all of them collected by the research team of the EpiReumatPt study.

Sample type

The sample type required for this project is one preparation of DNA from each subject. We estimate that 1 µg total DNA is sufficient to perform the assignment of the mtDNA haplogroups by using the combination of the Single Base Extension (SBE) assay and the PCR-RFLP technique.

We have no preferences in regards to frozen versus previously thawed samples, as long as the DNA integrity is acceptable. This can be ascertained in pilot studies.

Description of study participants

We will examine DNA from individuals in the EpiReumaPt cohort

Assays planned and methodology description

Our group published the methodology description to assign the most common European mtDNA haplogroups by means of the combination of the Single Base Extension (SBE) assay and the PCR-RFLP technique. Briefly, the SBE assay consists in the annealing of a single primer to a sequence of the mtDNA template that contains the SNP we want to interrogate, such that the

3'-end of this primer falls one base short of the SNP site present on the template. Since we are using only dideoxynucleotides (ddNTPs) in the reaction, when the complementary base is incorporated by the Taq DNA polymerase, the elongation stops and, depending on the fluorescence emitted, the SNP site will be identified. Prior to the SBE assay, a multiplex PCR is performed in order to amplify the six fragments that contain the 6 SNPs of interest. By means of the SBE assay, the 6 most common haplogroups (H, V, K, U, J and T) can be assigned in one multiplex reaction followed by a fragment analysis in an automatic sequencer with capillary technology; the remaining samples that are not assigned with this assay are processed by PCR-RFLP following the hierarchical scheme described by our group

Progression criteria

We will analyze the radiographic knee OA progression during the follow-up period according to the radiographic data available. Since all the participants in the study have been subjected to X-rays, we'll expect to analyze the influence of the mtDNA haplogroups on the radiographic progression in terms of KL grade, defining progression as an increase of at least one KL grade in either knee during the follow-up period. Additionally, we should also analyze the development or progression of i) joint space narrowing in the medial compartment (mJSN), ii) osteophytes in the tibia medial compartment and iii) subchondral sclerosis in the tibia medial compartment. The progression criterion for each of these three features will be an increase of at least one OARSI atlas grade in either knee. Additionally, if available, we should analyze the influence of the mtDNA haplogroups on the rate of structural progression over time by means of the change in radiographic joint space width (mm) in the medial compartment of both knees.

9. Análise estatística (statistics analysis) (Max: 150 palavras/words)

Deverá incluir uma descrição sumária da metodologia a efectuar de acordo com as características do(s) outcome(s) e avaliação das covariáveis e confundidores. Deverá incluir também a dimensão da amostra e o power do estudo.

Should include:

- . a brief description of the methodology according to the characteristics of the(s) outcome(s)
- . the assessment of covariates and confounders.
- . the sample size and the power of the study.

Prior to perform a multivariate analysis, a descriptive assay using SPSS will be carried out, including 2x2 contingency tables in order to analyze the frequency distribution of the mtDNA haplogroups between progressors and non-progressors.

Turnbull's extension of the Kaplan-Meier curve to interval-censored data will be used to estimate the cumulative probability of progression over time (survivor function) according to the mtDNA haplogroups. An extended Cox proportional hazard model using the iterative convex minorant algorithm will be used for multivariate analysis adjusting for the confounder effects of gender, age, BMI, functional indexes, life style, health status, morbidity, therapy and sociodemographic at baseline. Due to difficulties in deriving the asymptotic behavior of statistic tests based on interval-censored data, statistical significance will be tested by confidence intervals (CIs) for the hazard ratios (HR) by means of resampling methods. Therefore, CIs will be obtained using the bootstrap methodology (1000 replicates) with the percentile method. For this purpose, comparisons between haplogroups will be performed considering the most common mtDNA haplogroup, H, as the reference group.

Based on our previous studies, taking into account the frequency of the haplogroup J and T in the general population (~10%), and in order to detect a significant variation of 10% in the prevalence of the haplogroup J or T in progressors vs non-progressors group, we will need to analyze at least 900 samples of the EpiReumaPt subcohort to reach a confidence level of 95% and a statistical power of 80%.

10. Cronograma (Time line)

We will analyze DNA from 986 knee OA patients in the EpiReumaPt cohort and anticipate to complete assignment of the mtDNA haplogroups within 5 months. Data analysis will require 3 months so that the entire project can be completed in less than 1 year.

11. Orçamento (Budget)

We have financial support from the Profesor Novoa Santos Foundation (it is a Foundation associated to the Hospital Universitario A Coruña).

12. Publicações (deverá incluir a informação se os dados obtidos constituirão propriedade exclusiva do promotor; quem terá acesso aos dados e se a responsabilidade da publicação é da exclusiva responsabilidade do promotor):

Publications (including information of data property, who will have access to the data and if the promoter is the only responsible by the data publication):

(Max: 300 palavras/words)

The data property belongs to the promoters (mtDNA haplogroups study group and EpiReumaPt team) and they have the responsibility of the publications).

13. Estratégia de protecção dos dados do participante (Strategy of data protection) (Max: 300 palavras/words)

All samples will be descodified and data will be managed in sample groups without any reference to individual data.

14. Outras questões éticas relevantes (se aplicável) (Other relevant ethics issues, if applicable):

N/A.

Anexos:

- . Carta de apresentação
- . Consentimento Informado para recolha de amostras biológicas
- . Cv do IP do presente estudo