Official Title of the study: Mitoquines as prognostic factors of exacerbations and hospital admission in COPD patients

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INDEX

SUMMARY

The most important pathogenic factor of Chronic Obstructive Pulmonary Disease (COPD) in the Western world is chronic exposure to tobacco smoke, which induces oxidative stress not only in the respiratory system, but in all the body. Mitoquines (Humanin, MOTS-c, FGF21 and GDF15) are circulating hormones directly or indirectly produced by dysfunctional mitochondria, whose function is to protect the body of the consequences of oxidative stress. The objective of this project is to study the modifications that are produced in the serum mitoquines from patients with COPD of varying severity and to assess their potential applications in the clinic.

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is characterized by progressive and hardly reversible obstruction of the airways, which basically affects the small airways (chronic obstructive bronchiolitis), variably associated with destruction of the pulmonary parenchyma (emphysema) (1). 10% of people over the age of 45 years have COPD (2). COPD is expected to be the third leading cause of mortality in the world by 2020 (3). The leading cause of COPD in Western countries is chronic tobacco smoke contact with the airways, with massive entry of many toxic substances and reactive oxygen species (ROS) to the body that induce oxidative stress (OS) (4). OS in COPD is both exogenous (ROS inhaled) and endogenous (ROS induced by tobacco toxics and by the own disease). Some researchers consider that OS in COPD is closely related to a peculiar type of accelerated cellular senescence associated with a chronic inflammatory process ("inflammaging"), which not only affects to the respiratory system, but also many other parts of the body (skeletal muscle, cardiovascular system, global metabolism, immunological system, etc.) (5-12).

Mitochondrial dysfunction plays a central, but not exclusive, role in oxidative stress, cellular senescence and chronic inflammation. Therefore, a better understanding of the mitochondrial dysfunction underlying COPD would make possible to better understand the physiopathology and to identify new possible therapeutic targets. The mitochondrial alterations of COPD at the bronchopulmonary, muscular and immunological areas are widely documented both morphologically and pathophysiologically (13-27).

Mitochondrial dysfunction may be primary (congenital) or secondary (acquired, as in the case of tobacco smoking). It is a broad concept including impaired cellular energy production, excessive generation of ROS or of some metabolites from the Krebs cycle in the mitochondria, and loss of quality control of essential mitochondrial components that finally lead to abnormal output of intramitochondrial molecules (mtDNA, ATP, cytochrome c, Romo1 etc.) to the cytosol and extracellular fluids. Some of these molecules behave like DAMPs (damage associated molecular patterns) and induce an activation of innate immunity, and thus inflammation. Blood levels of Cytochrome C and Romo1 have been proposed as markers of oxidative stress (27).

The main function of the mitochondria is the generation of ATP, the basic

energy-carrying molecule for the maintenance of the living cell. The generation of ATP is produced from other energy precursors in the mitochondria through the oxidative phosphorylation system coupled to the electron transport chain (OXPHOS/ETC). These organelles also play a fundamental role in 1) the generation of specific metabolites of carbohydrates, lipids and proteins, which are essential for multiple cellular functions, 2) the synthesis of hemes and steroid hormones, 3) the management of the "clusters" of Fe and S, 4) cellular homeostasis of intracellular calcium, 5) immune response, both innate and acquired, and 6) the regulation of some types of apoptosis.

Cells react continuously to the environmental changes to which they are exposed (28). In situations of cellular stress (e.g. caloric deprivation, lack of specific nutrients, changes in temperature, etc.) cell nucleus reacts by sending signals to the mitochondria so that they
modify their function to adapt to the change (anterograde signaling from the nucleus to the mitochondria: for example physical exercise consumes ATP in the muscle cell, which activates the AMPK, which activates the nuclear transcription cofactor PGC-1alpha, which in turn activates OXPOS/ETC and mitochondrial biogenesis). On the other hand, when there is a stressful situation in the mitochondria themselves (e.g. excessive production of oxygen free radicals, unfolded intramitochondrial proteins, etc) signals are sent to the nucleus for it to modify the production of proteins intended to prevent/correct mitochondrial damage, including chaperones, antioxidants or proteolytic enzymes proteolytic (autonomic or intracellular retrograde signalling).

Mitochondria have recently been shown to be capable of directly or indirectly generate peptides that not only influence the own cell that produces them, but they have at distance effects(non-autonomous or extracellular retrograde signalling). These substances, discovered by Dillin's group (29,30), are called mitoquines and send signals from tissues with "stressed" mitochondria to the whole body, being hormones that "prepare" the whole organism to respond to the cellular stress it's going to be subjected to. Mitoquines are released when there is any kind of mitochondrial stress (congenital or acquired mutations in the mtDNA, disorders of the
OXPHOS/ETC that generate oxidative stress, mitochondrial toxins, etc.). In mammals mitochondrial stress is generally associated with the so-called "integrated cellular response to the stress (ICRS)". One of the most important mechanisms of ICRS is the UPR ("Unfolded protein response"), in which mitochondria participate in a coordinated way with
the endoplasmic reticulum system, and the cell nucleus (28,31-36).

There are at least two different types of mitoquines: 1) primary mitoquines,
encoded in mitochondrial DNA (mtDNA) and 2) secondary mytokines, encoded in the nucleus DNA (nDNA), whose secretion is regulated through activation signals from the "stressed" mitochondria to the nDNA (e.g. ATF4, etc) (33). Humanin (HN), MOTS-c ("Mitochondrial ORF of the Twelve S-c"), and six peptides similar to humanin (SHLPs 1-6) are considered primary mitoquines, although the number may be higher. Until recently, it was thought that mitochondrial DNA encoded only 37 genes (13 peptides found exclusively into the mitochondria, all of them sub-units of the ETC, 22 transfer RNAs -tRNA-, and 2 ribosomal RNAs -rRNA-) RNAs. We now know that 16s and 12s rRNA contain sORFs ("short open reading frames") that
translate secretory peptides from 20-30 aa. It is not known what intimate mechanisms regulate the synthesis and release of these mitoquines, although it is possible that they are related to a mitochondrial ribosomal activation. Regarding secondary mitoquines

(those encoded in nuclear DNA) under the activation of ATF4 we know fibroblastic growth factor 21 (FGF21), growth and development factor 15 (MIC1/GDF15), follistatin and intermedin-adrenomedullin2. These mitoquines also respond to other stimuli, independent of the mitochondria.

Humanin is a peptide of 21 or 24 aa, with multiple cytoprotective functions against mitochondrial damage (increases the synthesis of antioxidants and chaperones for unfolded proteins). It is anti-inflammatory (lowers the inflammatory cytokines and raises the
anti-inflammatory cytokines) and antiapoptotic (blocks apoptotic factors
such as Bak and IGFBP3), through at least 2 membrane receptors and several
interactions with other intracellular and extracellular proteins, in many tissues (nervous system, liver, heart, vascular wall, skeletal muscle, retinal pigment epithelium, gonads etc) (37-43). It also has beneficial metabolic effects (decreases insulin resistance at the central level, protects the pancreatic beta cell from oxidative stress and has negative feedback with IGF1) (44,45). Recently it has been proven that people with high levels of this hormone have less cognitive impairment with age (43) and are also very longevous (46). MOTS-c is another small peptide of 16 aa, encoded mtDNA, but synthesized exclusively in the cytosolic ribosomes, that also has beneficial antioxidant and metabolic effects, as it decreases insulin resistance and prevents obesity (40,47,48). On the other hand, it increases resistance against some infections increases (49) and decreases bone resorption, so it may have antiosteoporotic effects (50). Both hormones are measurable in the blood by ELISA, although there are certain discrepancies in their plasma levels depending on the test used.

FGF21 is a well-characterized hormone that stimulates ketogenesis and beta oxidation of fatty acids (51). Its secretion is regulated by fasting and activation of receptors PPAR-α, but it is also known that it rises in any situation of mitochondrial stress that activates mitochondria- to-nucleus signals (52,53). The MIC1/GDF15 is another circulating hormone that reduces hunger, activating a specific receptor level (GFRAL) found in the postrema area and the nucleus of the solitary tract (54,55). Elevated levels of GDF15 have been found in cancer cachexia and in many other situations, among them COPD (55,56). It is also released when the mitochondrial-to-nucleus signals are activated (57).

In COPD, of all the mitoquines reviewed here, there is only information regarding GDF15 blood levels, but there are no data in the literature regarding the levels of the other mitoquines. As COPD progresses, mitoquines blood levels are likely to increase progressively, expressing further deterioration of mitochondrial function, although their levels could increase only up to a certain level, and then decrease when mitochondrial damage is unbearable, thus constituting a kind of mitohormetic response (58).

HYPOTHESIS

Mitoquines, expressed in the context of mitochondrial dysfunction, are altered in COPD patients and are associated with worst clinical outcomes. Furthermore, mitoquines can be used as prognostic factors and potential therapeutic targets in COPD.

OBJECTIVES

1.- To describe mitoquines levels in a control group, a group of stable COPD outpatients and a group of exacerbated COPD patients.

2.- To describe differences in semitones levels in different groups of COPD patients (different levels of obstruction, patients with high risk of exacerbation vs. no risk of exacerbation, patients with CAT score<10 vs rest of patients, patients with low Fat-Free-Mass index (FFMI) vs. rest of the patients).

3.-To evaluate the correlation between semitones and different clinical outcomes such as FFMI, distance walked in 6 minute walking test, FEV1, CAT score.

4.- To evaluate if mitoquines can be used to predict future risk of exacerbation and hospital admission.

METHODS

Study population

Inclusion and exclusion criteria:

Stable COPD patients: will be selected from Pneumology outpatient clinics from Hospital Universitario Marqués de Valdecilla. Patients with COPD must meet the following criteria: 40 years or older with baseline post-bronchodilator forced expiratory volume in 1 s [FEV1]/forced vital capacity [FVC] ≤0.70.
Exacerbated COPD patients: Will be selected from patients admitted at Hospital Universitario Marqués de Valdecilla with the diagnosis of COPD exacerbation.
Control group: will be obtained from patients without COPD or any other acute or chronic respiratory condition and and patients' relatives.

Accepting an alpha risk of 0.05 and a beta risk of 0.2 in two-sided test 30 subjects are necessary in the first group and 90 in the second to find as statistically significant proportion difference expected to be of 0.45 in group 1 and 0.1 in group 2. Anticipating a drop-out rate of 5%. The ARCSINUS approximation. This calculation has been performed according to previously published studies performed by our group. We calculate a simple size of 120 patients with COPD, 30 patients with COPD exacerbation, and 30 controls.

Target enrollment/sample size 180
Anticipated rate of enrollment 25 patients each month

Estimated study start date: Samples collected in Biobank from 01.12.2019 Samples sent to biochemistry lab 01.03.2020
Estimated study completion date: (end of follow up) 05.04.2021

Study Design and methods

Observational prospective study.

Patients will be recruited from COPD outpatient clinics, Smoking cessation outpatient clinics and from patients hospitalized due to COPD exacerbation.

All patients will be given written informed consent to participate. This study was already approved by the Ethics Committee of Cantabria (CEIC).

Participants

1) Stable COPD (40 years or older with baseline post-bronchodilator forced expiratory volume in 1 s [FEV1]/forced vital capacity [FVC] ≤0.70) will be recruited during their regular follow-up.

2) Control group: age- and sex-matched volunteers without previous diagnosis of COPD or other respiratory conditions, and with post-bronchodilator forced expiratory volume in 1 s [FEV1]/forced vital capacity [FVC] >0.70.

3) Exacerbated COPD patients: Patients with previous diagnosis of COPD (40 years or older with baseline post-bronchodilator forced expiratory volume in 1 s [FEV1]/forced vital capacity [FVC] ≤0.70) admitted to hospital pulmonology Ward due to COPD exacerbation.

Charlson Comorbidity Index will be recorded from all participants in the study. Patients with acute exacerbations 1 month prior to the study, patients included in pulmonary rehabilitation during the study or 6 months before the inclusion period, with other potential causes of sarcopenia (malignant diseases, heart failure, hyperthyroidism or other chronic devastating diseases) and patients with known chronic kidney diseases or recent acute kidney injury will be excluded from the study.

Blood samples and all other measurements will be made the same day patients accept to enter the study.

Clinical Characteristics

At the enrollment in the study, COPD patients will be divided into different categorical groups: (1) non symptomatic patients (COPD Assessment Test [CAT] score < 10) versus symptomatic patients (CAT score ≥10), (2) non dyspneic patients (modified Medical Research Council dyspnea score [mMRC] < 2) versus dyspneic patients (mMRC ≥2), (3) high risk of exacerbation patients (those with 2 or more exacerbations requiring treatment with antibiotics or systemic steroids or at least one hospital admission in the previous year) versus low risk of exacerbation patients, and (4) former smokers versus active smokers.

After entering the study, blood samples will be obtained, and patients will be followed up for 1 year (one visit after 6 months and one visit after 12 months) and exacerbations and hospital admissions will be recorded prospectively. During the follow-up period, all clinical investigators in the study will be blinded to the mitoquines results. Along this period, patients with possible pulmonary exacerbations will be instructed to go freely to the Emergency Department of the Hospital and that team of doctors will freely decide to hospitalize them or not, according to their own clinical criteria.

According to the mitoquines levels patients will be divided into two groups: one composed of those within the highest quartile of the mitoquines and the other group will include patients in the other three quartiles of the levels of mitoquines.

Measurements

Basal Dyspnoea will be recorded using mMRC dyspnoea scale. CAT score will be recorded by self-administered questionnaire. Previous exacerbations will be recorded from

clinical records from patients included in the study. Spirometry will be measured according to the American Thoracic Society/European Respiratory Society (ATS/ERS) in all subjects. Body composition will be estimated by a bioelectrical impedance device (OMROM BF511, Omrom, Japan), and the FFMI will be calculated as the ratio of the FFM to the height in meters squared. The 6-min walking test will be performed according to the protocol of the American Thoracic Society: patients were asked to walk as far as they can in 6 min in a 30-m straight corridor without any interruption. At the end of the test, the distance walked by the patients and dyspnea will be recorded. Humanin and MOTS-c will be measured by ELISA (Mybiosource), FGF21 y GDF15. Will be measured by ELISA (R&D Quantikine). If possible Romo1 will be measured also by ELISA. The study will be divided in 3 visits:

VISIT 1: Blood sample collection and clinical characteristics.
VISIT 2: 6 months after visit 1: Exacerbations and hospital admissions (number and date) after visit 1.
VISIT 3: 12 months after visit 1: Exacerbations and hospital admissions (number and date) after visit 2.

Study endpoints

Primary endpoint: Mitoquines can be used to estimate hospital admission risk in COPD patients.

Secondary endpoints:

1.-Mitoquines can be used to estimate COPD exacerbation risk in COPD patients.
2.- Mitoquines are altered in COPD patients.
3.- Mitoquines are altered in COPD exacerbations.
4.- Mitoquines correlate with different COPD variables (FEV1, FFMI..).

Statistical plan or data analysis

Data will be presented as mean ± SD for normally distributed data or median (interquartile range) for nonparametric data. Differences between groups will be analyzed using unpaired t tests for parametric data or Mann-Whitney tests for nonparametric data. Correlations between data sets were examined using the Pearson (r) correlation coefficient for parametric data or the Spearman rank (rs) correlation coefficient for nonparametric data. Normal distribution will be tested using a Kolmogorov-Smirnov test. Kaplan-Meier estimates will be used to calculate the proportion of participants who have an event over time. Univariate and multivariate analysis using the Cox proportional risk analysis will be performed using SPSS Software version 25.00 for PC to analyze the development of the first events according to basal levels of mitoquines, and to identify risk factors associated with exacerbations and hospitalization. Differences will be considered significant if p values were less than 0.05. All reported p values will be two-sided.

Limitations and ethical considerations

This is a single-centre study thus, it must be replicated in multicentric studies, using a higher number of patients coming from different countries.
Although expensive and complicated, some techniques such as muscle biopsy, ergometry, muscle mass quantified using CT or shuttle test could be performed in order to have a better overview of muscle mass and function in these patients.

No potential harm for patients is expected from this study. This study was approved by Cantabria ethics committee (Code: :2018.276). Although the study is funded by the company GlaxoSmithKline (GSK) it is an independent study and the investigators do not receive any financial compensation.

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