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Looking into aqueous humor through metabolomics spectacles – exploring its metabolic characteristics in relation to myopia

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ABSTRACT

Aqueous humor is the transparent fluid found in the anterior chamber of the eye that provides the metabolic requirements to the avascular tissues surrounding it. Despite the fact that metabolomics could be a powerful tool in the characterization of this biofluid and in revealing metabolic signatures of common ocular diseases such as myopia, it has never to our knowledge previously been applied in humans. In this research a novel method for the analysis of aqueous humor is presented to show its application in the characterization of this biofluid using CE–MS. The method was extended to a dual platform method (CE–MS and LC–MS) in order to compare samples from patients with different severities of myopia in order to explore the disease from the metabolic phenotype point of view.

With this method, a profound knowledge of the metabolites present in human aqueous humor has been obtained: over 40 metabolites were reproducibly and simultaneously identified from a low volume of sample by CE–MS, including among others, a vast number of amino acids and derivatives. When this method was extended to study groups of patients with high or low myopia in both CE–MS and LC–MS, it has been possible to identify over 20 significantly different metabolite and lipid signatures that distinguish patients based on the severity of myopia. Among these, the most notable higher abundant metabolites in high myopia were aminooctanoic acid, arginine, citrulline and sphinganine while features of low myopia were aminoundecanoic acid, dihydro-retinoic acid and cysteinylglycine disulfide.

This dual platform approach offered complementarity such that different metabolites were detected in each technique. Together the experiments presented provide a whelm of valuable information about human aqueous humor and myopia, proving the utility of non-targeted metabolomics for the first time in analyzing this type of sample and the metabolic phenotype of this disease.

1. Introduction

Myopia, commonly referred to as short/near-sightedness is the condition that occurs when light entering the eye does not impact in the retina but in front of it, producing focused images when looking at close objects, but generating out of focus images when looking objects that are at a longer distance. An emetrope eye has an axial length and a power combination of the eye lenses (cornea and crystalline lens) that lead to a focused image in the retina. An emetrope eye, therefore, does not need glasses to see well. Myopia is one of the most frequent of the refractive eye defects. It can present in two forms: high myopia where there is an excessive axial length (greater than 26 mm) or low myopia when the axial length is less

Abbreviations: AHA, aqueous humor; CE–MS, Capillary electrophoresis - mass spectrometry; LC–MS, Liquid chromatography - mass spectrometry; D, diopters; IOP, intraocular pressure; QC, Quality control; BGE, background electrolyte; MFE, Molecular Feature Extraction; MT, migration time; RT, retention time; LOESS, Locally estimated scatter-plot smoothing; OPLS-DA, ortho partial least squares - discriminant analysis; VIP, Variable importance projection; ASS, argininosuccinate synthase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

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than 26 mm and there is a mismatch defect of the refractive elements (cornea or lens) that are unable to produce an image focused in the retina. Myopia can be diagnosed in any time of life; patients are normally diagnosed as children (between 8 to 12 years old) and their condition may worsen during adolescence, but myopia can also be diagnosed in adulthood due to visual stress or health conditions such as diabetes [1]. It is known that myopia can be caused by genetic factors; by 2012 almost 30 genes had been identified and associated to myopia [2]. The condition of myopia is considered to be one of the most common human eye diseases, affecting 18.5 % of Asians, 13.2 % of Hispanics, 4.4 % of Caucasians and 6.6 % of African Americans [1]. Since it is very common, the severity of myopia is not considered, although maculopathy, cataract, glaucoma and retinal detachment are some of the complications that myopia can lead to. Nevertheless, high myopia is one of the leading cause of blindness in adulthood in developed countries and is the last step of all these [1]. Therefore myopia is a topic that could lead to many studies in the near future.

Aqueous humor (AH) is a transparent fluid found in the anterior and posterior chamber of the eye. It is secreted by the ciliary epithelium lining the ciliary body. Three mechanisms are involved in aqueous humor formation: diffusion, ultrafiltration and active secretion. Active secretion is the major contributor to aqueous humor formation. The fluid is continuously secreted by the ciliary epithelium and enters first into the posterior chamber; then it travels through the pupil toward the anterior chamber and the trabecular meshwork passively traveling towards the episcleral venous system. The composition of AH depends not only on the nature of its production, but also on the metabolic interchanges that occur within various tissues throughout its intraocular route. It is responsible for the supply of nutrients and oxygen to the avascular tissues through diffusion [3]. The major components of AH are organic and inorganic ions, carbohydrates (glucose), urea, and proteins, oxygen, carbon dioxide and water. As AH is 'a transudate of plasma', provides the amino acids required for the synthesis of lenticular proteins [4]. Besides that, important anti-oxidant substances can also be found in the AH, such as glutathione (derived by diffusion from the blood) and ascorbate (which helps protect against light- induced oxidative damage) and defensive molecules such as immunoglobulins (IgG, IgM and IgA) [3].

AH also removes metabolic waste from the avascular tissues through its continuous formation, moving through the ocular chambers and drainage from the eye to the venous blood. In a healthy eye, the intraocular pressure (IOP) is necessary to inflate the eye as well as to maintain the proper shape and optical properties of the eyeball and AH secretion and outflow are responsible for maintaining IOP. Impaired outflow of AH results in elevation of the IOP, a central principle of glaucoma and its treatment [3].

AH (and more specifically AH in relation to myopia) has previously been studied using proteomics [5–8], but, despite its involvement on the metabolic interchanges throughout its intraocular route, myopia has never to our knowledge been studied through a metabolomics approach in humans. However, AH for different conditions has been previously studied through a metabolomics approach in murine models [9,10]. In this study, an untargeted method using capillary electrophoresis–mass spectrometry (CE–MS) metabolomics has been applied to characterize the profile of AH, chosen due to its applicability within the common platforms for metabolomic analysis to analyze ionic and more polar, water soluble compounds such as amino acids [11] known to be comprised in this type of sample [4]. In addition, the low sample volume consumption characteristic for CE results a great value when working with AH with a limited volume. Following this, AH samples from 36 patients with low (<6 D) myopia and high (≥7 D) myopia were analyzed using the same method in CE–MS and the analysis was extended to include liquid chromatography mass spectrometry (LC–MS), both with accurate mass analyzers in order to gain a wider coverage of metabolites in order to investigate the metabolic alterations with severity of myopia.

1.1. Materials and methods

1.1.1. Experimental design

The study consisted of several experiments. The first was focused on the study of the metabolic profile by CE–MS, whereby six independent preparations were obtained from a sample coming from a healthy individual. For the second experiment, metabolomics was applied to study patients with high or low myopia in order to gain insight into the mechanism of the condition.

1.2. Reagents

All the reagents and standards used were of analytical grade. The dilutions were made with ultrapure water obtained with a MilliQ® system (Millipore, Billerica, MA, USA). The internal standard, methionine sulfone (99%), methanol (MS quality) and formic acid (98%) were from Sigma- Aldrich (Steinheim, Germany). The reference masses used, Purine (121.0509) and HP0921 (922.0098), were obtained from Agilent Technologies (Santa Clara, CA, USA).

1.3. Samples

The samples of AH (50 μL) were kindly provided by the Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunitat Valenciana (FISABI O-Oftalmología Médica) in Valencia (Spain). AH samples were provided for myopic patients: 12 with high myopia (≥26 mm) and 24 with low myopia (< 26,5 mm) and one came from a patient without myopia (for method development). All of the AH samples were extracted during a cataract surgery. All patients provided written informed consent for samples to be taken, and the approval from the ethic committee was obtained for the study.

All samples were diluted with ultrapure water 1:5 (50 μL of sample and 250 μL of water) and were subsequently kept at −80 °C until the day of the experiment. On the day of the experiment, samples were defrosted and homogenized by vortex mixing.

Quality control (QC) samples were prepared by pooling equal volumes of each AH sample and were used in the analysis to observe stability and reproducibility. In both experiments (characterization of the profile and analysis of samples from high and low myopic patients), QCs were injected at the start to reach system stability. In the latter experiment, QCs were also injected every five samples in order to track reproducibility throughout the longer analytical run.

For CE–MS, samples were prepared by mixing 75 μL of the sample (processed as previously described) with 5 μL of internal standard solution resulting in a final concentration by dilution with the AH sample of 0.15 mM methionine sulfone and 0.10 M formic acid. For LC–MS, samples were centrifuged (4 °C, 10 min, 16000 *g*) and the supernatant was analyzed directly.

1.4. Metabolomic platforms

CE–MS analyses were performed on a 7100 capillary electrophoresis (Agilent Technologies) coupled to a 6224 accurate mass TOF MS (Agilent Technologies), equipped with an electrospray ionization (ESI) source. The separation was performed in a capillary with an inner diameter of 50 μm and a total length of 100 cm working in normal polarity. Before each analysis the capillary was conditioned with a background electrolyte (BGE) (0.8 M formic acid solution in 10 % methanol (v/v)) for 5 min at 950 mbar. Sample injections were performed for 50 s at 50 mbar. After the injection