

**ATP Level and Cough Sensitivity to ATP in Subjects with Refractory/Unexplained Chronic Cough**

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**Study Protocol**

## **Subjects and Study Design**

Patients with RCC/UCC were recruited in the Cough Clinic at the First Affiliated Hospital of Guangzhou Medical University from May 2022 to January 2024. According to Chinese guideline, RCC/UCC referred to a chronic cough: 1) without identifiable causes after throughout investigation; 2) persistent after empirical treatment to known causes; 3) persistent after treatment for positive conditions known to be associated with chronic cough<sup>1</sup>. The diagnosis of RCC/UCC strictly adhered to the criteria above. Other inclusion criteria included: 1)  $\geq 18$  years old; 2) cough as sole symptom lasting  $\geq 6$  months; 3) cough visual analogue score  $\geq 30$ mm. Age and gender matched healthy controls ( $\geq 18$  years old) would be recruited.

For all subjects, exclusion criteria were as follow: 1) smoking currently or in the past 6 months, or a smoking history of  $>20$  pack-year; 2) a forced expiratory volume in 1 second/forced vital capacity ratio  $<70\%$ ; 3) positive findings of chest radiography; 4) use of inhaled/oral corticosteroid, bronchodilators, anti-allergic medicine, antitussive medicine or anti-reflux treatment in the past 1 week; 5) a history of acute respiratory infection in the past 4 weeks; 6) a history of severe disease (cancer, acute coronary syndrome and so on). Additionally, any signs of cough hypersensitivity would lead to exclusion of healthy controls.

Clinical features recording, chest radiography, routine blood test, pulmonary function tests, induced sputum test, ATP cough challenge and capsaicin cough challenge were performed to all subjects. Supernatant of sputum were stored at  $-80^{\circ}\text{C}$  for the further ATP measurement. Capsaicin cough challenge was performed at least 30min after ATP cough challenge. All subjects were informed of the study procedures and provided written informed consent. The study was approved by the Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University (No. ES-2023-027-01).

## **ATP measurement in Induced sputum**

Sputum was induced and processed as described previously<sup>2</sup>. Briefly, sputum was induced with 3% saline. Sputum plugs were selected and mixed with four times its volume of 0.1% dithiothreitol. The cell smear was stained with hematoxylin-eosin. The differential cell count was obtained by

counting 400 non-squamous cells. Supernatant was stored in -80°C for the following measurement of ATP. The ATP level was measured using the ATPlite 1-Step Luminescence Assay System (Revvity, USA), following the provided instructions. The briefly procedure for using a 96-well microplate to measure ATP involves equilibrating the substrate and buffer at room temperature, reconstituting the lyophilized substrate solution with buffer, adding 100  $\mu$ L of the reconstituted reagent to each well containing 100 $\mu$ L induced sputum supernatant, shaking the microplate for 2 minutes, and then measuring luminescence.

### **Cough challenge**

ATP cough challenge was performed firstly and capsaicin cough challenge was following with an interval of at least 30min. ATP solutions (Sigma-Aldrich, USA) were prepared with 0.9% saline as solvent, with concentrations ranged from 0.1 mM, 0.316 mM, 1 mM, 3.16 mM, 10 mM, 31.6 mM, 100 mM and 316 mM. The solutions were stored in refrigerator at 4°C and were re-prepared every three days. Capsaicin cough challenge was performed with capsaicin (Sigma Aldrich, Seattle, USA) solutions beginning at a concentration of 1.95  $\mu$ M and then doubling the concentration up to 1000  $\mu$ M as described previously. Concentration–response challenges to inhaled ATP and capsaicin were undertaken with a compressed air driven nebulizer. Briefly, the single-breath dose-response method was adopted using a compressed air-driven nebulizer controlled by a breath-activated dosimeter (APS, JAEGER) whose output and duration of each nebulization were 160 mg/min (calculated as 0.9% saline) and 0.5 seconds, respectively. Coughs in the first 30 seconds after each inhalation recorded. The termination of cough challenge was the inhalation of the solution of highest concentration. The lowest concentration that provoked at least 2 coughs (C2) and the lowest concentration that provoked at least 5 coughs (C5) were recorded. For capsaicin, C5 of the healthy subjects was  $\geq$ 125  $\mu$ M, so hypersensitivity to capsaicin was defined as C5<125  $\mu$ M<sup>3</sup>.

1. [Chinese expert consensus on the diagnosis and treatment of chronic refractory cough]. Zhonghua Jie He He Hu Xi Za Zhi. 2021;44(8):689-98. doi: 10.3760/cma.j.cn112147-20210320-00186. PubMed PMID: 34645134.
2. Zhan W, Luo W, Zhang Y, Xiang K, Chen X, Shen S, Huang C, Xu T, Ding W, Chen Y, Lin M, Pan

X, Lai K. Sputum Transcriptomics Reveals FCN1+ Macrophage Activation in Mild Eosinophilic Asthma Compared to Non-Asthmatic Eosinophilic Bronchitis. *Allergy Asthma Immunol Res.* 2024;16(1):55-70. doi: 10.4168/aaair.2024.16.1.55. PubMed PMID: 38262391; PMCID: PMC10823142.

3. [Chinese national guideline on diagnosis and management of cough(2021)]. *Zhonghua Jie He He Hu Xi Za Zhi.* 2022;45(1):13-46. doi: 10.3760/cma.j.cn112147-20211101-00759. PubMed PMID: 35000304.