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TREATMENT WITH LONG ACTING MUSCARINIC ANTAGONISTS STIMULATES SERUM LEVELS OF IRISIN IN PATIENTS WITH COPD

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KEYWORDS: Chronic obstructive pulmonary disease (COPD)**;** Irisin **;** Long acting muscarinic receptor antagonist (LAMA) **;** Anticholinergics

ABSTRACT

Long acting muscarinic antagonists (LAMA) are currently considered the therapeutic mainstay for patients with COPD and have been shown to improve clinical outcomes including symptoms, exercise capacity and airflow limitation. Irisin, is a newly discovered hormone-like myokine generated by skeletal muscle cells in response to exercise and it is suggested to regulate energy expenditure and exercise capacity. The aim of the present study was to investigate if treatment with LAMA alters serum irisin levels in patients with COPD. Irisin was assessed by ELISA in the serum of 506 patients with COPD, GOLD II-IV, with a smoking history > 10 PY, who were included in the PROMISE-COPD cohort. The effect of inhaled LAMA on serum irisin levels was evaluated in a *proof-of-concept* cohort of 40 COPD patients.

Univariate linear regression analysis revealed that there was a significant negative association of irisin with age-adjusted Charlson score ($p = 0.003$) and a positive association of irisin with 6-min walking distance (6MWD) ($p = 0.018$) and treatment with LAMA ($p = 0.004$) but not with LABA or ICS. Multivariate analysis revealed that the association of irisin with LAMA treatment remains significant after adjustment for age-adjusted score and 6MWD. In the *proof-of-concept* cohort a single inhalation of LAMA stimulated serum irisin levels after 4h.

These findings imply that treatment of COPD patients with LAMA increase circulating irisin, thus explaining some of the beneficial extra-pulmonary effects of these drugs when used in the treatment of COPD.

ABBREVIATIONS: 6MWD, 6-min walking-distance; COPD, chronic obstructive pulmonary disease; LAMA, long acting muscarinic antagonists; FNDC5, fibronectin-type-III domain-containing protein-5; MMRC, Modified Medical Research Council Score; SGRQ, the St. George's Respiratory Questionnaire (SGRQ)-COPD version

[1] The two first authors contributed equally to the study.

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Introduction

Long-acting muscarinic antagonists (LAMA) in combination with long-acting beta2-agonists are increasingly being used to treat chronic obstructive pulmonary disease (COPD) due to greater improvement in lung function and symptom scores [1-5]. Both classes of bronchodilators relax airway smooth muscle and thereby reduce respiratory muscle activity and dynamic hyperinflation and improve ventilatory mechanics [6-8]. In patients with COPD, the use of LAMA, as compared to placebo, reduced lung hyperinflation and inspiratory capacity at rest and during exercise contributing to improvement in exertional dyspnea and increased exercise endurance [8]. Beside their bronchodilatory effects, LAMA, as compared with LABA, have been shown to exert superior anti-inflammatory activity reducing the production of superoxide and pro-inflammatory mediators in COPD patients [9]. However, the beneficial effects of LAMA for COPD patients cannot be based solely on their bronchodilatory and their anti-inflammatory actions [10-12]. LAMA have been shown to increase exercise endurance in patients with COPD, with an as yet unknown mechanism, other than reduction of lung hyperinflation [13].

Irisin is a newly described myokine generated by the degradation of the muscle cell protein assigned as fibronectin type III domain-containing protein 5 (FNDC5) [14,15]. FNDC5 expression and serum irisin are increased by exercise and therefore both proteins are regarded as indicators for increased muscle activity and improved endurance [16,17]. In COPD, serum irisin has been correlated with exercise capacity [18,19]. Additional factors, such as diet or insulin metabolism, have been shown to be dominant regulators of irisin release, while gender and age are less important [20].

In the present study, we investigated if treatment with LAMA alters serum levels of irisin in a cohort of 506 COPD patients. We further explored the effect of LAMA on irisin regulation in a *proof-of-concept* cohort of 40 COPD patients.

Methods

STUDY DESIGN, PATIENTS

The study cohort consisted of 638 patients enrolled in the "PRedicting Outcome using systemic Markers In Severe Exacerbations of Chronic Obstructive Pulmonary Disease" (PROMISE-COPD) study, a multicenter study in 11 centers in 8 European countries. The PROMISE-COPD study was designed to be inclusive, exploratory and hypothesis generating. It was specifically drafted to identify predictors of outcome using systemic markers in patients with moderate to very severe COPD [forced expiratory volume in 1 s (FEV1) less than 80% of predicted value after bronchodilator use and a ratio of FEV1 to forced vital capacity (FVC) of 0.7 or less after bronchodilator use (grade II-IV)], based on physical examination and spirometry, at least 4 weeks after the latest exacerbation was resolved. All patients were older than 40 years, current or ex-smokers with a smoking history of ≥10 pack-years. Exclusion criteria were rapid fatal disease with death expected within 6 months, pulmonary condition other than COPD as the main respiratory disease, immunosuppression including organ transplantation or chronic steroid use (> 20 mg prednisolone equivalent per day) and muscle-skeletal or neuromuscular process preventing ambulation.

All enrolled patients had an initial baseline examination at stable state and were followed up for at least two years in scheduled visits every six months. When necessary, patients underwent outpatient visits or were hospitalized for treatment of AECOPD, and follow up visits were performed 4 weeks after the onset of exacerbation.

Throughout the study duration, patients were treated as clinically warranted, without restriction. Patients were monitored for recurrent moderate (requiring treatment with

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systematic corticosteroids, antibiotics, or both) and severe AECOPD (requiring hospitalization or a visit to the emergency department). COPD exacerbation was defined as an acute change from baseline in dyspnea, cough, and/or sputum production beyond normal day-to-day variation that necessitates use of antibiotics, glucocorticoids, or both.

Clinical history, physical examinations, lung function, and 6-min walking test (6MWT) were performed for each patient. The age-adjusted Charlson Comorbidity Index Score was also calculated. Each patient also completed the Modified Medical Research Council Score (MMRC), the St. George's Respiratory Questionnaire (SGRQ)-COPD version and the Short Form-36 (SF-36) health related, quality of life questionnaire. All examinations took place at each scheduled visit (semi-annually), except for the 6MWT. At the 2-year follow up, the vital status of each patient was confirmed.

We also included a *proof-of-concept* cohort of 40 COPD patients who participated in the N_2COPD Washout study (N_2 washout for evaluation of small airway involvement in COPD) [21], to test the effect of LAMA inhalation on serum irisin levels. These patients did not receive treatment with LAMA for 24 h or 48 h and then they inhaled a single dose of LAMA (glycopyrronium 44 mcg or tiotropium, 18 mcg). Serum samples were collected before and then 1 h and 4 h after the inhalation of LAMA.

The PROMISE-COPD study and the N_2COPD Washout study were investigator-initiated and driven studies compiled with the Helsinki Declaration and GCP Guidelines, and were approved by the Institutional review Board (EKBB295/07 and EKNZ340/13, respectively). The PROMISE-COPD study was registered at www.controlled-trials.com (identifier ISRCTN99586989). All patients provided written consent for the study assessments.

DETERMINATION OF IRISIN

Serum irisin was determined by ELISA (Adipogen, Liestal, Switzerland). All samples were assayed in duplicate.

Statistics: For the patient cohort, the Null-hypothesis was that there was no difference of serum irisin between patient groups, clinical parameters or treatment. The data were compared using a computer assisted statistic program SPSS (version 220 for Macintosh, IBM, Switzerland). For all statistics p-value < 0.05 was considered as significant. Continuous variables are expressed as the mean $\pm$ SD or median (interquartile range: 25th to 75th percentile) and discrete variables as percentage. Linear univariate and multivariate regression model analysis was applied to evaluate the relationship between irisin and clinically relevant variables in COPD.

Results

PATIENTS

From the 638 patients that were enrolled in the study, a total of 506 patients attended a scheduled visit at 6 months. These patients were followed-up for a period of 722 [395-762] days. During the follow-up, 317 patients (62.6%) suffered from one or more exacerbations (354 exacerbations in total) and 38 patients (7.5%) deceased (Fig. 1).

Most patients in the PROMISE-COPD cohort were male, with a considerable smoking history and clinically relevant disease (1 exacerbation requiring physician attention in the previous year) (Table 1) and multiple comorbidities (online Table 1). Characteristics of the 354 exacerbations are presented in online Table 2.

ASSOCIATION OF SERUM IRISIN WITH BASELINE DEMOGRAPHIC AND CLINICAL CHARACTERISTICS

Univariate linear regression analysis revealed that serum irisin was inversely associated with

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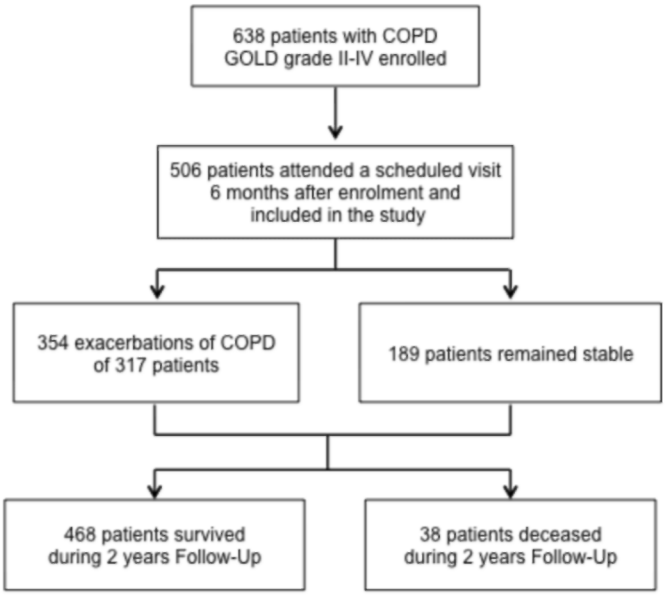
age-adjusted Charlson score (p = 0.003) and positively correlated with: (a) 6MWD (p = 0.018); (b) the emotional role in SF-36 questionnaire (p = 0.034) and (c) treatment of patients with LAMA (p = 0.004) (Table 2). The association of irisin with the age-adjusted score and LAMA remained significant (p = 0.008 and p = 0.001, respectively) after adjustment for 6MWD and emotional role in the SF-36 questionnaire, as revealed by multivariate linear regression analysis (Table 3).

LAMA INCREASE SERUM IRISIN

Patients with stable COPD, who were receiving LAMA, had higher levels of irisin (median: 1.4 ± 0.1 μg/ml), as compared with patients who were not receiving LAMA (median: 1.0 ± 0.1 μg/ml) (p < 0.001) (Fig. 2).

To validate the effect of LAMA on serum irisin levels, we measured irisin in the serum of 40 COPD patients, with stable disease, who were included in the *proof-of-concept* cohort. The baseline characteristics of these patients are presented in Table 4. Patients were instructed to stop the inhalation of LAMA for 24 h (n = 23) or 48 h (n = 17) prior the experiment (Fig. 3). Serum irisin was determined before LAMA inhalation, and 1 h and 4 h after a single inhalation of LAMA. In patients that were on 24-h LAMA withdrawal, we did not observe any effect of LAMA on serum irisin levels neither 1 h nor 4 h after LAMA inhalation. However, in patients that did not receive LAMA for 48 h, serum irisin was not altered 1 h after LAMA inhalation but increased significantly 4 h after LAMA inhalation (p = 0.047) (Fig. **4).**

Fig. 1*. Strengthening the Reporting of Observational studies in Epidemiology (STROBE) flow chart for the patients in the PROMISE-COPD cohort. PROMISE-COPD: Predicting Outcome Using Systemic Markers in Severe Exacerbations of Chronic Obstructive Pulmonary Disease; GOLD: Global Initiative for Chronic Obstructive Lung Disease.*



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Discussion

Irisin is a recently described myokine, mainly released by SkMC, which has been proposed to mediate the beneficial effects of exercise [14,22, 23]. Up to date, there is very limited evidence regarding the role of irisin in COPD [18,19,24]. In the present study, serum irisin levels were assessed in a large cohort of 506 patients with COPD, at stable state and at exacerbation. The data provide evidence, for the first time, that serum irisin in COPD patients at stable state, was inversely associated with the age-adjusted Charlson score, while positively associated with the 6MWD and treatment with LAMA. Furthermore, in a *proof-of-concept* cohort, it was demonstrated that inhalation of LAMA increased serum irisin in COPD patients.

In COPD patients at stable state, serum irisin was inversely associated with age-adjusted Charlson score, indicating that low serum irisin levels are related with the number and severity of comorbidities. The most common comorbidity was arterial hypertension (50.6% of the patients), followed by coronary arterial disease (22.5%), congestive heart failure (15%) and diabetes mellitus (12.3%). Previous studies have shown a negative association between circulating irisin levels and type-2 diabetes mellitus [25,26]. However, the regulation of irisin in humans, as well as the role of irisin in glucose metabolism remains unclear, with conflicting results for the association of irisin and glucose levels, insulin levels, and insulin resistance [26,27].

Serum irisin levels at stable state did not correlate with age, BMI or with BODE index, which is in agreement with a study showing that serum irisin concentration did not correlate with age, BMI, glucose, insulin, lipoproteins, cholesterol metabolism or adipokines [28]. In our cohort, serum irisin was associated with 6MWD, indicating that high serum irisin is related to exercise capacity in COPD patients. Multivariate analysis showed that the association of irisin with 6MWD was lost when adjusted for the age-adjusted Charlson score, indicating that comorbidities have a greater impact on exercise capacity in COPD patients. Exercise has been associated with increased irisin levels [29]. Greulich et al. [24] showed that whole body vibration exercise in hospitalized patients with COPD was associated with increased levels of irisin and improvement in 6MWD whereas in the study of Ijiri et al., it was demonstrated that acute exercise did not affect serum irisin, but eight-week exercise training was linked to significant increase in its levels [18].

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***Table 1**. Baseline characteristics of 506 patients with COPD GOLD II-IV, at stable state, included in the PROMISE-COPD cohort.*

Characteristics		n = 506
Age, years		66.8 ± 10.5
Gender, Male, No. (%)		366 (71.9)
BMI, kg/m^2		26.2 ± 5.5
Weight, kg		74.9 ± 17.0
Height, cm		169.0 ± 8.0
White, yes (%)		501 (99.0)
Current smoker no. (%)		150 (29.6)
Pack-years, years		51.5 ± 30.9
Duration of COPD symptoms, months		102.7 ± 89.1
Time elapse since diagnosis, months		80.2 ± 74.6
MMRC dyspnea scale		1 [1-2]
BODE index		3 [1-4]
6MWD, meters		380.3 ± 104.2
Borg Score		4 [3-6]
SaO_2 at rest, %		94.5 ± 2.7
Lowest SaO_2 at exercise, %		89.5 ± 5.7
Heart rate at rest, bpm		80.4 ± 14.5
Highest heart rate at rest, bpm		104.8 ± 18.4
SGRQ		
Symptoms score		49.0 ± 22.7
Activity score		57.4 ± 22.8
Impact score		32.2 ± 18.7
Total Score		42.4 ± 18.1
SF-36		
Physical function		51.4 ± 25.9
Role Physical		51.7 ± 43.5
Role emotional		67.5 ± 42.9
Social functioning		69.8 ± 28.2
Mental health		65.4 ± 19.8
Body pain		73.9 ± 27.6
Vitality		51.9 ± 20.9
General Health		48.2 ± 23.1
GOLD grade[a], n %		253 (50.0)
	II	
	III	177 (35.0)
	IV	76 (15.0)
FVC, post-brd, % predicted		77.8 ± 24.8
FEV_1, post-brd, %predicted		48.6 ± 18.2
FEV_1/FVC post-brd, %		48.2 ± 14.1
Residual volume (RV), %		157.4 ± 45.6
Total lung capacity (TLC), %		118.8 ± 20.21
RV/TLC, %		53.7 ± 9.6
Diffusion capacity (D_{LCO}), %		55.6 ± 20.7
Arterial blood gas analysis		
PaO_2, mm Hg		69.0 ± 51.8
$PaCO_2$, mm Hg		39.8 ± 6.8
Therapy for COPD		
LAMA		343 (67.7)
LABA		369 (72.9)
ICS		402 (79.4)
Systemic glucocorticoids		22 (4.3)

Continuous data are shown as mean ± SD or median IQR, and categorical variables as No. (%). BMI: body mass index; Age-adjusted score: Age-adjusted Charlson Comorbidity score; 6MWD: 6-min walk distance; bpm: beats/min; brd: bronchodilator; D_{LCO}: Diffusion capacity of the lung for carbon monoxide; GOLD: Global Initiative for Chronic Obstructive Lung Disease; ICS: Inhaled corticosteroids; MMRC: modified Medical Research Council; LABA: Long-acting beta2-agonist; LAMA: Long-acting muscarinic antagonists; $PaCO_2$: Carbon dioxide pressure; PaO_2: Oxygen pressure; SABA: short-acting beta2-agonist; SaO_2: Peripheral oxygen saturation; SF-36: Short Form-36; SGRQ: St. George's Respiratory Questionnaire.

[a] GOLD grades are based on FEV_1% predicted: II, > 50% < 80%; III > 30% < 50%; IV, < 30%. There were no patients with GOLD grade I COPD because of study inclusion criteria.

***Table 2.** Univariate linear regression analysis for the association of serum levels of irisin with different treatments in patients with COPD (n = 506), included in the PROMISE-COPD cohort.*

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Log IRISIN	β	95% CI	P value
LABA	-0.218	-0.963-0.527	0.566
LAMA	0.861	0.279-1.442	0.004
ICS	-0.329	-1.025-0.367	0.353
Systemic steroids	0.066	-1.258-1.390	0.922

LABA: Long-acting beta2-agonist; LAMA: Long-acting muscarinic antagonists; ICS: inhaled corticosteroids.

Table 3. *Multivariate linear regression model for the association of irisin with COPD characteristics in a cohort of 506 patients included in the PROMISE study.*

Variable	β	p value
Age-adjusted Charlson score	-0.248 (-0.430--0.066)	0.008
6 min walking distance	0.002 (-0.001-0.005)	0.142
Role emotional	0.004 (-0.003-0.010)	0.258
Treatment with LAMA	0.967 (0.376-1.559)	0.001

LAMA: Long-acting muscarinic antagonists.

Fig. 2. *Serum irisin levels in COPD patients at stable state who were not receiving LAMA (n = 152) and who were receiving LAMA (n = 354).*

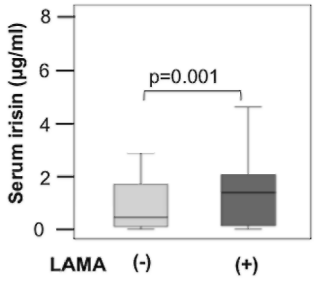

Table 4. *Baseline characteristics of 40 patients with COPD GOLD II-IV, at stable state, included in the proof-of-concept cohort.*

Characteristics		n = 40
Age, years		66.7 ± 16.0
Gender, Male, No. (%)		25 (62.5)
Weight, kg		74.9 ± 17.0
Height, cm		166.8 ± 8.3
GOLD grade[a], n %	I	2 (5.0)
	II	26 (65.0)
	III	12 (30.0)
FVC, post-brd, % predicted		86.4 ± 16.7
FEV_1, post-brd, %predicted		56.1 ± 15.6
FEV_1/FVC post-brd, %		47.4 ± 11.3
Residual volume (RV), %		144.5 ± 41.5
Total lung capacity (TLC), %		107.7 ± 18.5
RV/TLC, %		51.7 ± 9.4
Diffusion capacity (D_{LCO}), %		59.9 ± 15.8

Continuous data are shown as mean ± SD or median IQR, and categorical variables as No. (%). GOLD: Global Initiative for Chronic Obstructive Lung Disease.

[a] GOLD grades are based on FEV_1% predicted: I > 80%; II > 50% < 80%; III > 30% < 50%.

In this study, serum irisin was associated with treatment of patients with LAMA and this association remained significant after adjustment for other factors that were also associated with irisin such as the age-adjusted Charlson score, 6MWD and the emotional role in the SF-36 questionnaire. LAMA belongs to first-line treatment in patients with COPD with proven

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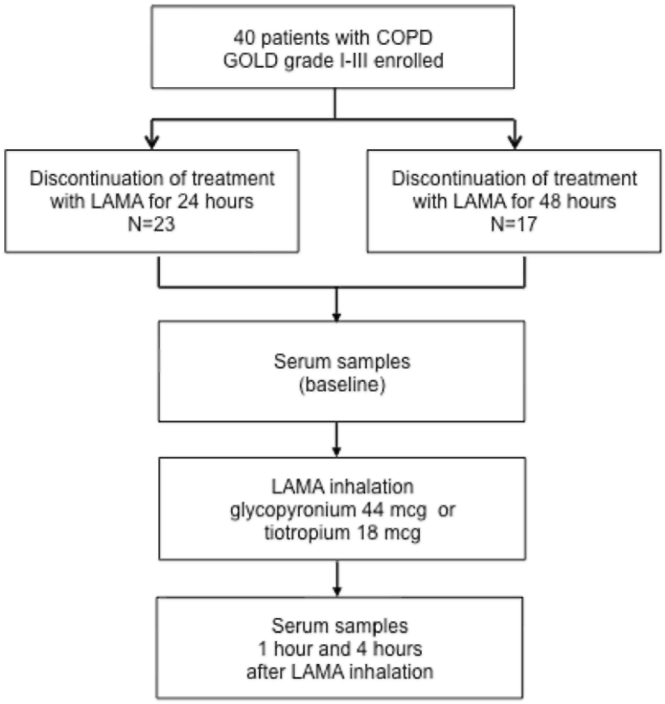
beneficial therapeutic outcomes. In this study, treatment with LAMA, but not with any other medication for COPD, was associated with serum irisin levels. Patients with stable COPD, who were receiving LAMA, had higher serum levels of irisin, as compared with patients who were not receiving LAMA. Despite the fact that there is an overlap of irisin values between the two groups of patients, the fact that: (a) measurements were always performed in duplicate; (b) the large number of patients in each group (354 patients that were receiving LAMA and 152 patients that were not receiving LAMA) and (c) the high intra-assay and inter-assay precisions, with coefficient of variation 5.7% and 9.6%, respectively, convinces for the robustness of the assay.

This is the first time that the effect of LAMA on irisin has been investigated *in vivo,* in a small *proof-of-concept* cohort, including 40 COPD patients. The inhalation of LAMA increased serum irisin between one and four hours after inhalation. This effect was significant only in patients that did not inhale LAMA for 48 h prior the experimental administration of LAMA, indicating that the effect of LAMA on irisin secretion remains for longer than 24 h. These findings fit very well with the recent documentation that treatment with tiotropium, at a daily dose of 5 μg, resulted in a significant improvement in exercise tolerance in patients with moderate COPD, while treatment with indacaterol, at a daily dose of 150 μg, did not, although no significant differences were observed between the two treatments regarding their effects on lung hyperinflation, exercise-related dyspnea, and daily-life dyspnea [13]. This clearly indicates that other mechanisms than sustained reductions of lung hyperinflation should be considered to explain improved exercise tolerance after treatment with LAMA. Since it was shown that serum irisin levels were associated with the level of physical activity in COPD patients, and that an eight-week exercise training was linked to a significant increase in irisin level [18], the increased serum irisin levels after a single inhalation of LAMA documented in the present study may be considered the piece of information missing.

Fig. 3. *Strengthening the Reporting of Observational studies in Epidemiology (STROBE) flow chart for the patients in the proof-of-concept cohort. GOLD: Global Initiative for Chronic Obstructive Lung Disease.*



COPD is associated with various extra-pulmonary manifestations including cachexia and muscle atrophy [30]. It has been shown that skeletal muscle functions like strength and endurance, as well as muscle structure like fibre quality, capillary density and metabolic capacity, are altered in patients with COPD [31]. Furthermore, it has been shown that during acute exacerbations, the anabolic-catabolic ratio is shifted towards the catabolic state of the muscle, which is

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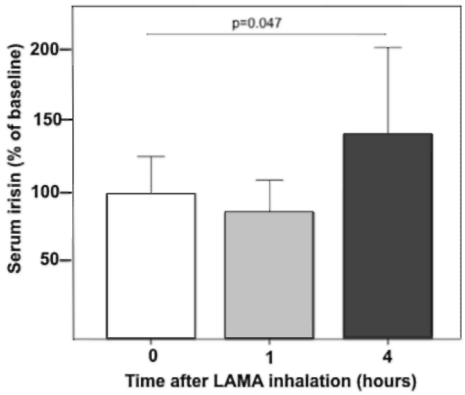
accompanied by ischemia-related apoptosis injury [30]. Considering that irisin increases total body energy expenditure and resistance to obesity-linked insulin resistance [26] and that administration of irisin has been proposed as a potential therapeutic target to treat obesity and diabetes, it may be postulated that the stimulatory effect of LAMA on irisin release observed *in vivo* and *in vitro* in this study, underlines an additional beneficial effect of LAMA in mediating metabolic restoration and increasing exercise capacity in COPD patients.

Fig. *4. Serum irisin levels in COPD patients, at stable state, who did not receive LAMA for 48 h (n = 17), 1 h and 4 h after a single dose of LAMA inhalation. Results are presented as % over baseline value that is irisin levels before the LAMA inhalation, p-value was calculated by the Friedman test for all three values.*



The present study must be interpreted within the context of certain limitations. In the proof-of-concept cohort, cessation of LAMA treatment for 24 and 48 h was probably inadequate to achieve total wash out of the drugs. However, the fact that we observed a significant increase of irisin, 4 h after the administration of LAMA, as compared to baseline that was under the influence of the remaining LAMA, indicates that our conclusion for the significant stimulation of irisin by LAMA is solid. It remains to be established whether the magnitude of irisin increase after LAMA inhalation is associated with the clinical improvement or COPD prognosis. Nevertheless, the current data suggest a so far unrevealed extra-pulmonary mechanism mediating the action of LAMA in COPD.

In conclusion, treatment with LAMA increases serum irisin by a mechanism that involves shedding from its precursor molecule FNDC5 and this may mediate the additional beneficial effects of LAMA in COPD.

AUTHOR CONTRIBUTIONS

DS, MR, EP, JM conceived and designed the study, and analyzed the data. DS, WB, FB, RL, BM, KK, JA, GR, AM, AT, TW, LB, JR, AS, MT collected patient's data. JM, MR, and LC, performed cell biological experiments for drug's mechanism of action, and Enzyme Linked Immunosorbent Assay (ELISA). DS, and JM, conducted the statistical analyses. QS performed the Real-time quantitative PCR (RT-qPCR). All authors contributed to and approved the final manuscript taking complete responsibility for the integrity of the work from initiation until the publication.

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CONFLICT OF INTEREST STATEMENT

The authors have declared that no conflict of interest exists.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pupt.2017.10.011.

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