

Which is the best protocol and cut-off point in the 4-metre gait speed test to discriminate exercise capacity in COPD?

Vanessa Yumi Kozu Tino¹, Andrea Akemi Morita^{1,2}, Gianna Waldrich Bisca^{1,2} Giovana Guzzi¹ D, Felipe Vilaca Cavallari Machado¹ D, Nidia Aparecida Hernandes¹ D, Fabio Pitta¹ , Josiane Marques Felcar^{1,3}

- 1. Laboratório de Pesquisa em Fisioterapia Pulmonar, Departamento de Fisioterapia, Universidade Estadual de Londrina, Londrina (PR) Brasil.
- 2. Departamento de Fisioterapia, Centro Universitário Filadélfia, Londrina (PR)
- 3. Centro de Pesquisa em Ciências da Saúde, Universidade Pitágoras Unopar, Londrina (PR) Brasil.

Submitted: 12 July 2019. Accepted: 17 October 2019.

Study carried out in the Laboratório de Pesquisa em Fisioterapia Pulmonar, Departamento de Fisioterapia, Centro de Ciências da Saúde, Universidade Estadual de Londrina, Londrina (PR)

ABSTRACT

Objective: To determine the discriminative capacity and cut-off point of different 4-metre gait speed test (4MGS) protocols in identifying preserved or reduced exercise capacity using the six-minute walk test (6MWT) in patients with Chronic Obstructive Pulmonary Disease (COPD); also, to compare 4MGS protocols and characteristics of individuals according to the best cut-off point. Methods: We evaluated fifty-six patients with COPD, all of which were submitted to the assessment of anthropometric characteristics, pulmonary function (spirometry) and functional exercise capacity (6MWT and four protocols of the 4MGS). In the 4MGS test, patients were instructed to walk at normal pace and at maximum speed in a 4 meters course (4MGS 4m - usual pace and at maximum) and 8 meters course (4MGS 8m - usual pace and at maximum). Results: Only the 4MGS 4m-maximum protocol was able to identify preserved exercise capacity in the 6MWT (AUC=0.70) with moderate correlation between them (r=0.52; P=0<0.0001). The cut-off point found in the 4MGS 4m-maximum was 1.27 m/s. Patients with preserved exercise capacity (4MGS 4m-maximum ≥1.27m/s) walker greater distances on the 6MWT in %pred (91±2 vs 76±3; P<0.0001). In the other comparisons involving gender, BMI, FEV1% pred and GOLD index there were no significant differences between the groups. In addition, the agreement of individuals classified as preserved and reduced exercise capacity in the 6MWT and 4MGS 4m-maximum was significant (P = 0.008). Conclusion: The 4MGS 4m-maximum test can be used to discriminate preserved exercise capacity in patients with COPD and correlates with the 6MWT.

Keywords: Chronic Obstructive Pulmonary Disease; Velocity measurement; Gait.

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is defined as "[...] a common, preventable and treatable disease, characterized by persistent respiratory symptoms and limited airflow, due to airway abnormalities caused by significant exposure to harmful particles or gases"(1). In addition to airflow obstruction, COPD can also be characterized by deconditioning and physical inactivity. (2,3) Skeletal muscle dysfunction is an extrapulmonary characteristic of the disease, related to the decreased functional capacity for exercise and consequently to physical inactivity. Physical inactivity leads to physical deconditioning and is considered a risk factor for exacerbations and early mortality.(4-6) Therefore, the assessment of exercise capacity in patients with COPD is necessary for both the research field and clinical practice. (7)

Functional exercise capacity can be validated through field tests such as a six-minute walk test (6MWT).(7) The 6MWT is a simple, safe, low-cost test, easy to apply,

and reproducible, (7,8) in which the patient is instructed to walk the longest distance possible in a 30-meter flat corridor in six minutes. (8) This test enables us to evaluate global responses and integrated different systems involved during the exercise. (9) Also, a percentage of the predicted value below 82% is indicative of reduced exercise capacity in these patients.(10)

Although the 6MWT is considered a practical and simple test, it requires space (e.g. a 30-meter corridor), time (e.g. two tests with a 30-minute interval are needed) and trained personnel to apply the test. (7) Thus, alternative tests such as the 4-meter gait speed test (4MGS) have been used in patients with COPD to assess functional capacity.(11-14) In 4MGS, the patient must walk 4 meters at the speed requested to measure gait speed. Recent studies have shown an association between gait speed and exercise capacity in patients with COPD. (14,15) The 4MGS is considered a simple, reliable test that requires a small

Josiane Margues Felcar. Laboratório de Pesquisa em Fisioterapia Pulmonar, Departamento de Fisioterapia, Universidade Estadual de Londrina, Av. Robert Koch, 60, Vila Operária, CEP 86038-350, Londrina, PR, Brasil. Tel.: 55 43 3371-2477. E-mail: josianefelcar@gmail.com

Financial support: None.



space, is a low-cost, easy test to apply and quick to perform. $^{(12\text{-}14,16,17)}$

This test uses different protocols. The most frequently used are the 4MGS-4meter and the 4MGS-8meter, in which the patient is instructed to walk at his usual or maximum speed, crossing corridors of 4 and 8 meters. (12-14) However, differences between these protocols are yet unknown in terms of which of the 4MGS test protocols have the greatest discriminative power, and which cut-off value is capable of identifying patients with preserved or reduced exercise capacity assessed using the 6MWT.

The study aimed to verify the discriminatory power and cut-off point of the 4MGS protocols to identify the preserved or reduced exercise capacity in the 6MWT in patients with COPD; and to compare 4MGS protocols and the characteristics of the individuals according to the best cut-off point found.

METHODS

This is a cross-sectional study with a convenience sample of patients diagnosed with COPD, who were evaluated at the *Laboratório de Pesquisa em Fisioterapia Pulmonar* (LFIP) at the *Universidade Estadual de Londrina*, Londrina, PR, Brazil. All patients were under initial evaluation for inclusion in a pulmonary rehabilitation program unrelated to this study.

The Research Ethics Committee Involving Human Beings of the institution approved the study under CEP/UEL 080/2014. All participants signed an informed consent form.

The study included patients diagnosed with COPD, according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD),⁽¹⁾ with clinical stability, absence of comorbidities that could influence the performance of the tests and have not participated in physical training programs in the past twelve months. Individuals who for some reason were unable to complete all assessments were excluded from the study.

All patients underwent an anthropometric, pulmonary function and functional exercise capacity assessment. The anthropometric assessment was performed to better characterize the sample. Thus, the scale and stadiometer measured the weight and height (Ítaca Com. Equip. LTDA, model MIC2/BA, São Paulo - SP), determining the body mass index (BMI).

A portable spirometer (Spiropalm®; COSMED, Italy) was used for the lung function assessment. The technique was performed according to the guidelines of the American Thoracic Society/European Respiratory Society, $^{(18)}$ determining the forced expiration volume in the first second (FEV $_{\rm 1}$), forced vital capacity (FVC), and the FEV $_{\rm 1}$ /FVC index. We used the reference values proposed for the Brazilian population by Pereira et al. $^{(19)}$

Patients were also assessed for their functional exercise capacity through the 6-minute walk test (6MWT) and four different protocols of the 4-meter gait speed test (4MGS). The 6MWT was performed according

to internationally recommended standards, in which patients were instructed to walk the longest distance possible in a 30-meter corridor for six minutes. (7) Two tests were performed with an interval of at least 30 minutes between them and the longest walking distance was used for analysis. We used reference values described by Britto et al., (20) specific to the Brazilian population.

We used the calculation of the lower limit to classify individuals with preserved and reduced exercise capacity in the 6MWT. This calculation is performed by subtracting the product from 1.645 by the standard error of estimating the total distance equation predicted by the reference equation. (21)

In the 4MGS test, patients were instructed to walk at their usual pace and at maximum speed over 4 and 8 meters. The tests followed the protocols of Karpman et al.(12,13) and Kon et al.(14) In the 4MGS-4 meter protocol, the patient was instructed to walk 4 meters, at his usual pace and at maximum speed in a 4-meter corridor bounded by two cones. The stopwatch was started from the patient's first movement and was interrupted when the patient crossed the second cone.(14) In the 4MGS-8 meters, the individual was instructed to walk 4 meters at a usual pace and at fast speed but in a course of 8 meters: besides the 4 meters, the course had 2 meters of acceleration and 2 meters of a slowdown. However, the counting occurred only in the central 4 meters, discarding the initial 2 meters and the final 2 meters.(12,13) For each test, two repetitions were performed, and the test with the shortest execution time was used for analysis.

Statistical analysis

The Statistical Package for the Social Sciences software, version 20.0 (SPSS Inc., Chicago, IL, USA) and the GraphPad Prism, version 6.0 (GraphPad Software Inc., La Jolla, CA, USA) performed statistical analyzes. We also used the Shapiro-Wilk test for the analysis of the normality of the data. Results were described as mean and standard deviation or median and interquartile range 25-75% according to the data distribution. Also, we used the Pearson or Spearman correlation coefficient to verify the relationship between the 6MWT and the 4MGS protocols. The unpaired t-test or Mann-Whitney test compared the individuals with high and low performance on the 6MWT. The comparison of the 4MGS protocols was performed using the paired t-test or Wilcoxon test. For effect size analysis, we used the average of the difference in speed of the two protocols by the standard deviation of one of the protocols. The ROC Curve verified the discriminatory power of the 4MGS in identifying a preserved or reduced exercise capacity in the 6MWT and therefore, finding a cut-off point. The Chi-square test compared the proportion of individuals classified with preserved and reduced exercise capacity in the 4MGS and the 6MWT. Kappa verified the agreement of classification in the 6MWT and 4MGS, and the level of statistical significance was established as P < 0.05.



The sample power of this study was calculated according to the correlation results obtained between the 6MWT and the 4MGS test 4 meters maximum of r=0.52. Considering an alpha = 0.05 and a sample of 56 individuals, we obtained 99% power. The analysis was performed in the GPower software (Franz Faul, Universitat Kiel, Germany).

RESULTS

The study included 58 patients; however, two were excluded for not having completed all the proposed tests. Therefore, the data of 56 patients were considered for the analyzes, and Table 1 described general characteristics.

When the 4MGS protocols were compared, there was a difference between the tests performed on different corridor sizes, one with 4 meters distance and the other with 8 meters. The individuals walked at a higher speed in the 8-meter course, both at maximum speed and at the usual pace (Table 2). The effect size analysis found that when comparing the 4MGS protocols of usual pace and at maximum speed, the values obtained were 0.24 and 0.22, respectively. Also, a moderate correlation of the 6MWT distance with the 4MGS protocols 4 meters Maximum and 4MGS 8 meters Maximum was verified (r = 0.52 and r = 0.58, respectively; P < 0.0001 for both). A weak correlation was found between the 4MGS protocols 4 meters Usual (r = 0.33; P = 0.01) and 4MGS 8 meters Usual (r = 0.25; P = 0.05) and the 6MWT distance (Figure 1).

In the discriminative analysis, only the 4MGS 4 meters Maximum protocol identified the preserved exercise capacity in the 6MWT (AUC = 0.70) (Figure 2). However, none of the 4MGS protocols discriminated against a reduced exercise capacity in the 6MWT.

The cut-off point found in the 4MGS 4 meters Maximum was 1.27 m/s with a sensitivity of 0.750 and specificity of 0.625. When comparing the characteristics of the individuals according to the cut-off point found, patients with preserved exercise capacity (4MGS 4 meters

Table 1. General characteristics of individuals with COPD.

Table 1. General Characteristics of Individuals with COPD.		
Variables	N = 56	
Gender (M/F)	29/27	
Age (years old)	68 ± 8	
BMI (Kg/m²)	26 ± 5	
FEV ₁ (liters)	1.25 ± 0.44	
%FEV ₁ (%predicted)	50 ± 18	
FEV ₁ /FVC	55 [45-63]	
GOLD (I/II/III/IV)	1/32/16/7	
6MWT (m)	452 ± 73	
% 6MWT (%predicted)	85 ± 15	
4MGS 4 Meters Maximum (m/s)	1.36 ± 0.24	
4MGS 4 Meters Usual (m/s)	1.06 ± 0.23	
4MGS 8 Meters Maximum (m/s)	1.68 ± 0.31	
4MGS 8 Meters Usual (m/s)	1.29 ± 0.24	

Maximum ≥1.27m/s) were younger (65 [60-72] years old vs 72 [69-77] years old; P = 0.0016) and reached a greater distance covered in the 6MWT in absolute values $(485 \pm 56 \text{ meters vs } 395 \pm 65 \text{ meters; } P < 0.0001)$ and in the predicted% of the 6MWT (91 \pm 2 vs 76 \pm 3; P <0.0001). When comparing gender, BMI, FEV1% predicted and GOLD, no significant differences were observed between groups. Additionally, the agreement of individuals classified with preserved and reduced exercise capacity in the 6MWT and 4MGS 4 meters Maximum was significant (P = 0.008). Similarly, there was a greater proportion of individuals (83%) classified with 4MGS 4 meters Maximum ≥ 1.27 m/s as having exercise capacity preserved also by the 6MWT than in individuals classified (50%) as 4MGS 4 meters Maximum <1.27 m/s (P = 0.01) (Figure 3).

DISCUSSION

In this study, a cut-off point of 1.27 m/s was found in the 4MGS 4 meters Maximum test to discriminate preserved exercise capacity in the 6MWT. When comparing the characteristics of patients who had a speed above or below 1.27 m/s in the 4MGS 4 meters Maximum, those who had exercise capacity preserved by the 4MGS had a greater distance in the 6MWT in predicted %. In patients who presented walking speed above the cut-off point, 83% also had exercise capacity preserved by the 6MWT. When comparing the protocols, the individuals have a higher gait speed in the protocols performed in 8-meter corridors.

The 4MGS is considered an attractive test for clinical practice because it is simple, requires a small space, is inexpensive and easy to apply, and, therefore, it is used as an alternative and more practical way to assess functional capacity. (12-14,16,17) Several protocols are available in the literature, including in the study by Kon et al., (14) who used the 4-meter protocol with a specific characteristic: walking a 4-meter course with the usual walking speed. Karpman et al. (12,13) proposed the 4-meter walking protocol on an 8-meter course, with usual pace and at maximum speed. The 8-meter protocol features an acceleration zone of 2 meters, an area of 4 meters for timing, and a deceleration zone of 2 meters. In other studies, much more differentiated protocols are found, as in the study by Andersson et al. (22) In their study, patients were instructed to walk in a 30-meter corridor. First, they walked the course at a self-selected pace, and after a 2-minute rest period, they walked at maximum walking speed. Thus, the patients had a higher speed in the 4MGS performed in 8-meter corridors. This better performance in a larger corridor, both at usual pace and at maximum

Table 2. Comparison of 4-meter gait speed test protocols.

4MGS	4MGS	P
4 meters	8 meters	
1.06 ± 0.23	1.29 ± 0.24	<0.0001
1.36 ± 0.24	1.68 ± 0.31	<0.0001
	4 meters 1.06 ± 0.23	4MGS 4MGS 4 meters 8 meters 1.06 ± 0.23 1.29 ± 0.24 1.36 ± 0.24 1.68 ± 0.31

4MGS: 4-meter gait speed; P: p-value.



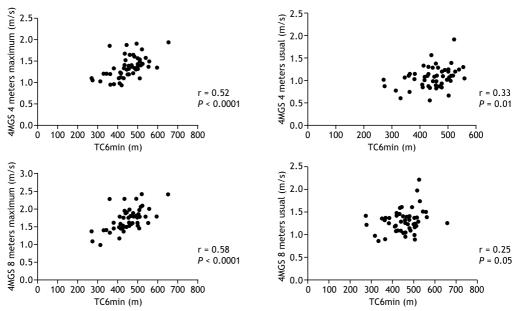


Figure 1. Correlation between the protocols of the 4-meter gait speed test (4MGS) and the six-minute walk test (6MWT). *P*: p-value; r: correlation coefficient.

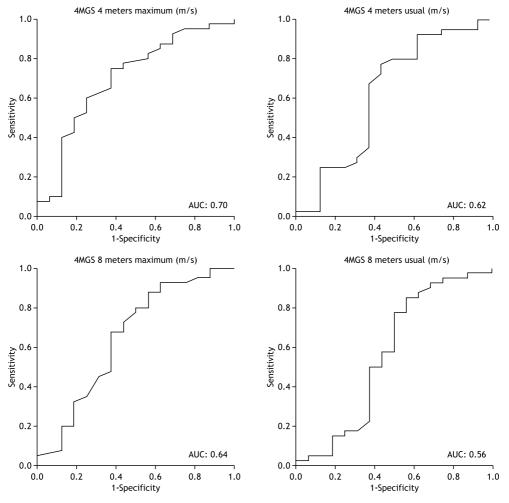


Figure 2. ROC curves of the different protocols of the 4-meter gait speed (4MGS) in meters per second (m/s) to identify preserved exercise capacity in the six-minute walk test. AUC: area under the ROC curve.

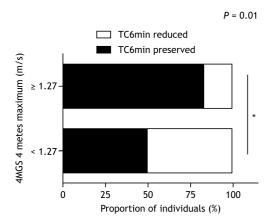


Figure 3. Comparison of the proportion of individuals with preserved exercise capacity in the six-minute walk test (6MWT) who walked at a speed greater or equal and less than 1.27m/s in the 4-meter gait speed of 4 meters - maximum speed (4MGS 4 meters maximum). *P*: p-value; *: *P*=0.01.

speed, can be explained by the zone of acceleration and deceleration that the protocol proposes. Timing starts when the individual is already moving and stops, without having to slow down, as there are still two meters (deceleration zone) to be walked.⁽¹³⁾

Among the variables studied, the 4MGS 4 meters and 8 meters Maximum showed a moderate correlation with the 6MWT. These findings corroborate other studies on the same topic. Karpman et al. (13) showed that gait speed is associated with exercise capacity (6MWT), and established that the correlation between gait speed at usual pace and at maximum speeds and 6MWT was high, regardless of the protocol (usual or maximum) (r = 0.77; r = 0.80, respectively; P <0.001). DePew et al.(15) also determined that 4MGS at the usual pace is significantly associated with the 6MWT (r = 0.70; P < 0.001). Finally, Kon et al. (14) also related the 4MGS at the usual pace with exercise capacity measured differently, using the Incremental Shuttle Walking Test, and found a positive and significant correlation between both tests (r = 0.78; P < 0.001).

Regarding the discriminative capacity of the 4MGS functional test, the maximum 4-meter protocol was the only one capable of identifying preserved exercise capacity in the 6MWT (AUC = 0.70). A possible explanation for this result may be due to the maximum speed being the one that best correlates with the exercise capacity since the 6MWT patients are encouraged to reach the longest distance possible. The Maximum 8-meter protocol had a lower discriminative capacity, which may be due to disregarding acceleration and deceleration. This difference may have occurred, as this aspect is not considered in the 6MWT since patients perform this acceleration and deceleration when turning the cones during the test. This study also found, in a novel way, the cut-off point in the 4MGS of 1.27m/s as identifying preserved exercise capacity by the 6MWT. Therefore, it is possible to use this speed obtained through a test simple as the 4MGS to screen those with preserved exercise capacity.

A limitation of this study is the convenience sample and the shortage of patients with a mild degree of airflow obstruction (GOLD I), which may compromise the external validity of the results for this population. However, individuals with a mild degree of the disease are prone to be asymptomatic and often do not seek treatment. Therefore, future studies are needed to investigate aspects of the 4MGS in these patients and for the cut-off point found in this study to be tested on other samples. Our findings can be used in future studies to screen patients with preserved exercise capacity more quickly and simply.

We concluded that the walking speed of 1.27 m/s obtained through the 4MGS 4 meters Max test identified a preserved exercise capacity in the 6MWT in patients with COPD. The 4MGS 4 meters maximum is correlated with the distance covered in the 6MWT.

ACKNOWLEDGMENTS

To all collaborators at the *Laboratório de Pesquisa em Fisioterapia Pulmonar* (LFIP) of the *Universidade Estadual de Londrina* (UEL) for the incentive to carry out this study.

REFERENCES

- GOLD: Global Initiative for Chronic Obstructive Lung Disease. Executive Committee. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. Bethesda: GOLD; 2019.
- Hernandes NA, Teixeira DC, Probst VS, Brunetto AF, Ramos EM, Pitta F. Perfil do nível de atividade física na vida diária de pacientes portadores de DPOC no Brasil. J Bras Pneumol. 2009;35(10):949-56. http://dx.doi.org/10.1590/S1806-37132009001000002. PMid:19918626.
- Pitta F, Troosters T, Spruit MA, Probst VS, Decramer M, Gosselink R. Characteristics of physical activities in daily life in chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2005;171(9):972-7. http://dx.doi.org/10.1164/rccm.200407-8550C. PMid:15665324.
- Ramon MA, Ter Riet G, Carsin A-E, Gimeno-Santos E, Agustí A, Antó JM, et al. The dyspnoea-inactivity vicious circle in COPD: development and external validation of a conceptual model. Eur Respir J. 2018;52(3):1800079. http://dx.doi.org/10.1183/13993003.00079-2018. PMid:30072504.
- Garcia-Aymerich J, Lange P, Benet M, Schnohr P, Antó JM. Regular physical activity reduces hospital admission and mortality in chronic obstructive pulmonary disease: a population based cohort study. Thorax. 2006;61(9):772-8. http://dx.doi.org/10.1136/thx.2006.060145. PMid:16738033
- Yohannes AM, Baldwin RC, Connolly M. Mortality predictors in disabling chronic obstructive pulmonary disease in old age. Age Ageing. 2002;31(2):137-40. http://dx.doi.org/10.1093/ageing/31.2.137. PMid:11937477.
- Holland AE, Spruit MA, Troosters T, Puhan MA, Pepin V, Saey D, et al. An official European Respiratory Society/American Thoracic Society technical standard: field walking tests in chronic respiratory disease. Eur Respir J. 2014;44(6):1428-46. http://dx.doi.org/10.1183/09031936.00150314. PMid:25359355
- Hernandes NA, Wouters EFM, Meijer K, Annegarn J, Pitta F, Spruit MA. Reproducibility of 6-minute walking test in patients with COPD. Eur Respir J. 2011;38(2):261-7. http://dx.doi.org/10.1183/09031936.00142010. PMid:21177838.



- ATS Committee on Proficiency Standards for Clinical Pulmonary Function Laboratories. ATS statement: guidelines for the six-minute walk test. Am J Respir Crit Care Med. 2002;166(1):111-7. http://dx.doi. org/10.1164/ajrccm.166.1.at1102. PMid:12091180.
- Troosters T, Gosselink R, Decramer M. Six minute walking distance in healthy elderly subjects. Eur Respir J. 1999;14(2):270-4. http://dx.doi. org/10.1034/j.1399-3003.1999.14b06.x. PMid:10515400.
- Karpman C, Benzo R. Gait speed as a measure of functional status in COPD patients. Int J Chron Obstruct Pulmon Dis. 2014;9:1315-20. PMid:25473277.
- Karpman C, Lebrasseur NK, Depew ZS, Novotny PJ, Benzo RP. Measuring gait speed in the out-patient clinic: methodology and feasibility. Respir Care. 2014;59(4):531-7. http://dx.doi.org/10.4187/ respcare.02688. PMid:23983271.
- Karpman C, DePew ZS, Lebrasseur NK, Novotny PJ, Benzo RP. Determinants of gait speed in COPD. Chest. 2014;146(1):104-10. http://dx.doi.org/10.1378/chest.13-2017. PMid:24522522.
- Kon SS, Patel MS, Canavan JL, Clark AL, Jones SE, Nolan CM, et al. Reliability and validity of the 4-metre gait speed in COPD. Eur Respir J. 2013;42(2):333-40. http://dx.doi.org/10.1183/09031936.00162712. PMid:23222875.
- DePew ZS, Karpman C, Novotny PJ, Benzo RP. Correlations between gait speed, six-minute walk, physical activity, and self-efficacy in severe chronic lung disease. Respir Care. 2013;58(12):2113-9. http://dx.doi. org/10.4187/respcare.02471. PMid:23696689.
- Ilgin D, Ozalevli S, Kilinc O, Sevinc C, Cimrin AH, Ucan ES. Gait speed as a functional capacity indicator in patients with chronic obstructive

- pulmonary disease. Ann Thorac Med. 2011;6(3):141-6. http://dx.doi.org/10.4103/1817-1737.82448. PMid:21760846.
- Bisca GW, Fava LR, Morita AA, Machado FVC, Pitta F, Hernandes NA. 4-Meter Gait Speed Test in Chronic Obstructive Pulmonary Disease: interrater reliability using a stopwatch. J Cardiopulm Rehabil Prev. 2018;38(4):E10-3. http://dx.doi.org/10.1097/HCR.00000000000000297. PMid:29251854
- Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. Eur Respir J. 2005;26(2):319-38. http://dx.doi.org/10.1183/09031936.05.0003 4805. PMid:16055882.
- Pereira CAC, Sato T, Rodrigues SC. New reference values for forced spirometry in white adults in Brazil. J Bras Pneumol. 2007;33(4):397-406. http://dx.doi.org/10.1590/S1806-37132007000400008. PMid:17982531.
- Britto RR, Probst VS, Andrade AFD, Samora GAR, Hernandes NA, Marinho PEM, et al. Reference equations for the six-minute walk distance based on a Brazilian multicenter study. Braz J Phys Ther. 2013;17(6):556-63. http://dx.doi.org/10.1590/S1413-35552012005000122. PMid:24271092.
- Culver BH. How should the lower limit of the normal range be defined? Respir Care. 2012;57(1):136-45. http://dx.doi.org/10.4187/ respcare.01427. PMid:22222132.
- Andersson M, Moberg L, Svantesson U, Sundbom A, Johansson H, Emtner M. Measuring walking speed in COPD: test-retest reliability of the 30-metre walk test and comparison with the 6-minute walk test. Prim Care Respir J. 2011;20(4):434-40. http://dx.doi.org/10.4104/ pcrj.2011.00082. PMid:21938352.