HHS Public Access

Author manuscript

Angiology. Author manuscript; available in PMC 2019 May 01.

Published in final edited form as:

Angiology. 2018 May ; 69(5): 416–423. doi:10.1177/0003319717726934.

ENDOTHELIAL CELL INFLAMMATION AND ANTIOXIDANT CAPACITY ARE ASSOCIATED WITH 6-MINUTE WALK PERFORMANCE IN PATIENTS WITH SYMPTOMATIC PERIPHERAL ARTERY DISEASE

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Abstract

We determined whether 6-min walk total distance and pain-free distance were associated with circulating biomarkers of antioxidant capacity and inflammation, and with cultured endothelial cell inflammation, oxidative stress, and apoptosis in 251 patients with symptomatic peripheral artery disease (PAD). In multivariate analyses, pain-free distance during the 6-min walk test was negatively associated with dyslipidemia ($p<0.001$), chronic kidney disease ($p=0.004$), and transient transfection, nuclear factor K-Light-Chain-Enhancer of activated B (NF-κB) cultured endothelial cells ($p=0.007$), and was positively associated with height ($p=0.008$). Furthermore, total distance walked during the 6-min walk test was negatively associated with cultured endothelial cell NF-κB ($p<0.001$), coronary artery disease ($p=0.009$), and body mass index ($p=0.022$), and was positively associated with ankle-brachial index ($p<0.001$), male sex ($p<0.001$), and hydroxyl radical antioxidant capacity ($p<0.001$). The 6-min walk performance in symptomatic patients with PAD was associated with vascular biomarkers, as walking distances were negatively associated with cultured endothelial cell inflammation and positively associated with circulating antioxidant capacity. The clinical implication is that behavioral interventions designed to alleviate endothelial cell inflammation and increase circulating antioxidant capacity, such as exercise and antioxidant intake, may improve ambulation of patients with PAD during submaximal exercise that is typically performed during daily activities.

Keywords

Antioxidant Capacity; Endothelial Cell; Exercise; Inflammation; Intermittent Claudication; Peripheral Artery Disease

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INTRODUCTION

Peripheral artery disease (PAD) is a prevalent condition,[1–3] that is both costly[4–7] and deadly.[8,9] Additionally, PAD is associated with poor patient-perceived quality of life, ambulatory leg pain,[10] impaired physical function,[11,12] reduced daily activity,[13,14] and faster declines in functional performance and mobility over time than subjects without PAD.[11,12,15] The 6-min walk test has been commonly used to assess walking impairment during submaximal exercise intensity in patients with PAD.[2,16] The 6-min walk distance is a clinically meaningful outcome measure in patients with PAD,[16] that is reliably measured at baseline,[17] sensitive to declines in untreated patients who are followed annually,[12] increases following a program of exercise rehabilitation,[18–20] and is associated with daily activity level.[21,22]

We have previously found that biomarkers of inflammation and oxidative stress are associated with walking dysfunction measured during a maximal treadmill test in symptomatic patients with PAD.[23] Possible mechanisms are that chronically high levels of inflammation and oxidative stress generated from ischemic lower extremities leads to muscle breakdown and damage,[24–26] and to acceleration of atherosclerosis,[27] both of which could contribute to poor maximal treadmill walking. However, potential muscle breakdown and acceleration in atherosclerosis from chronic inflammation may have even more clinical relevance for patients when they must perform submaximal exercise tasks to successfully complete activities associated with daily living. Previous work has found higher levels of circulating inflammatory biomarkers were associated with poor performance of 6-min walk distance in patients with PAD who were either asymptomatic or not limited in exercise by intermittent claudication.[28,29] However, less is known about patients with more severe PAD who are limited by intermittent claudication. It is not clear whether submaximal exercise performance, as measured by 6-min walk distance, in these more severely affected PAD patients is associated with circulating biomarkers of inflammation, and it is not known whether circulating antioxidant capacity may also have a positive influence. Furthermore, little is known whether biomarkers that are more specifically related to endothelial cell inflammation, oxidative stress, and apoptosis, may have even stronger associations with 6-min walk performance than circulating biomarkers.

The aim of the study was to determine whether 6-min walk total distance and pain-free distance were associated with circulating biomarkers of antioxidant capacity and inflammation, and with cultured endothelial cell inflammation, oxidative stress, and apoptosis in patients with symptomatic PAD. We hypothesized that shorter 6-min walk total distance and pain-free distance are associated with lower levels of circulating antioxidant capacity, and with higher levels of circulating inflammatory biomarkers and with greater cultured endothelial cell inflammation, reactive oxygen species (ROS) production and apoptosis.

METHODS

Patients

Approval and Informed Consent—The procedures of this study were approved by the institutional review board at the University of Oklahoma Health Sciences Center (HSC). Written informed consent was obtained from each patient at the beginning of investigation.

Recruitment—Vascular labs and vascular clinics from the University of Oklahoma HSC and the Oklahoma City VA Medical Center referred patients for possible enrollment into an exercise rehabilitation program to treat leg pain secondary to PAD.[18]

Baseline Clinical Assessments

Protocol—Patients were evaluated in the Clinical Research Center (CRC), at the University of Oklahoma HSC. Patients arrived before 9:00 am fasted, but were permitted to take their usual medications. To begin the study visit, patients underwent a medical history and physical examination by study physicians, in which claudication history, co-morbid conditions, cardiovascular risk factors and current medications were recorded. Following this assessment, study nurses obtained blood samples for fasting blood chemistry analyses. Samples were collected in vacutainers, distributed in 0.5 aliquots, stored at −80°C, and then batched for analysis.[23,30–32] Exercise personnel obtained demographic and anthropometric information,[33] and completed ankle-brachial index (ABI)[34] and exercise tests. Based on this evaluation, patients were coded on cardiovascular risk factors and co-morbid conditions according to standard definitions as previously reported.[35] The Homeostasis Model Assessment-Insulin Resistance (HOMA-IR) was calculated from the fasting plasma glucose and insulin levels.[36]

Inclusion and Exclusion Criteria—Patients were included if they screened positive for PAD by having all of the following criteria: (a) a history of ambulatory leg pain, (b) ambulatory leg pain confirmed by treadmill exercise,[10] and, (c) an ABI ≤ 0.90 at rest[2] or ≤ 0.73 after exercise.[37] Patients were excluded for any one of the following conditions: (a) absence of PAD (ABI > 0.90 at rest and ABI > 0.73 after exercise), (b) non-compressible vessels (ABI > 1.40), (c) asymptomatic PAD, (d) recent use of medications indicated for claudication (cilostazol or pentoxifylline) initiated within 3 months prior to investigation, (e) exercise limited by other diseases or conditions, (f) active cancer, (g) end stage renal disease defined as stage 5 chronic kidney disease, and, (h) abnormal liver function.

Outcome Measurements

6-min Walk Test—Patients performed an over ground, 6-min walk test in which 2 cones were placed 100 ft apart in a marked corridor.[17] Trained exercise technicians supervised the test and instructed the patients to walk as many laps around the cones as possible. During the test, patients indicated if and when they experienced the onset of claudication pain. The pain-free and total distances walked during the test were recorded in ft, and subsequently converted to m. The test-retest intraclass reliability coefficient is R = 0.75 for the pain-free distance, and R = 0.94 for the total 6-min walking distance.[17]

ABI—After 10 min of supine rest, the ankle and brachial systolic blood pressures were obtained as previously described.[38] Briefly, ankle systolic pressure was measured by Doppler technique in the posterior tibial and dorsalis pedis arteries of both legs. The higher of the two arterial pressures from the more severely diseased leg was recorded as the resting ankle systolic pressure. Similarly, brachial blood pressure was taken from both arms, and the arm yielding the higher systolic pressure was recorded as the brachial systolic pressure. The ABI was then calculated as ankle systolic pressure/brachial systolic pressure. The test-retest intraclass reliability coefficient for the measurement of ABI in our laboratory is R = 0.96 for ABI.[10]

Circulating biomarkers of inflammation and anti-oxidant capacity

High Sensitivity C-Reactive Protein (hsCRP): Concentration of hsCRP was quantified using a high-sensitivity Near Infrared Particles Immunoassay. A commercially available device, the SYNCHRON LX-20 (Beckman-Coulter; California, USA), was used to perform the assay.[39]

Serum Antioxidant Capacity: Hydroxyl Radical Antioxidant Capacity (HORAC) was measured using the OxiSelect HORAC Activity Assay (Cell Biolabs Inc., San Diego, CA) to determine the capacity of antioxidant enzymes and other redox molecules to counterbalance the deleterious effects of oxidative stress.[32]

Endothelial Cell Culture Bioassay: We used a cell culture-based bioassay approach utilizing cultured primary human arterial endothelial cells to characterize the endothelial effects of circulating factors present in the sera. Endothelial cells (purchased from Cell Applications, Inc., San Diego, CA, after passage 4; ages of the donors are unknown) were initially cultured in MesoEndo Endothelial Cell Growth Medium (Cell Applications, Inc) followed by Endothelial Basal Medium supplemented with 10% fetal calf serum until the time of serum treatment, as described.[40–43]

Apoptosis Assay: Cultured endothelial cells were treated for 24 h with sera of patients. To determine whether circulating factors in the sera exerted pro-apoptotic effects, apoptotic cell death was assessed by caspase activities using Caspase-Glo 3/7 assay kit (Promega, Madison, WI) as previously reported.[44,45]

Cellular Reactive Oxygen Species (ROS) Production: To assess cellular oxidative stress induced by factors present in the sera, hydrogen peroxide (H_2O_2) production in detector endothelial cells was measured fluorometrically using the Amplex Red/horseradish peroxidase assay as described.[46,47]

Transient Transfection, nuclear factor K-Light-Chain-Enhancer of Activated B (NF-κB) Cultured Endothelial Cells Reporter Gene Assay: To assess cellular pro-inflammatory effects induced by factors in the sera, transcriptional activity of NF-κB was tested in serum-treated detector endothelial cells by a reporter gene assay as described.[48]

Statistical Analyses

To summarize the clinical characteristics, continuous variables were presented as means and standard deviations, and binary variables were presented as percentages.

Univariate analyses were performed to assess the association between outcome variables (6-min Walk Pain-Free Distance and 6-min Walk Distance) and each clinical characteristics using linear regression for continuous variables and ANOVA for binary variables. Clinical characteristics with p-values <0.2 in the univariate analysis were considered as candidate variables for subsequent linear model variable selection. A stepwise procedure with Schwarz Bayesian Criterion (SBC) as implemented in SAS PROC GLMSELECT was performed for variable selection. The final models were then used to analyze the 6-min Walk Pain-Free Distance and 6-min Walk Distance.

All statistical analyses were performed using SAS 9.4. Statistical significance was defined by a two-sided p <.05.

RESULTS

The clinical characteristics of 251 patients with claudication are shown in Table 1. On average, the group consisted of older patients who were on the borderline between being overweight and obese. The group had a similar distribution of men and women, and Caucasians and African-Americans. The mean ABI and 6-min walk pain-free and total walking distances were all impaired, consistent with patients with claudication. As expected, there was a high prevalence of cardiovascular risk factors and comorbid conditions, with more than 80% of the patients having dyslipidemia, hypertension and metabolic syndrome.

The associations among the 6-min walk distances and the clinical characteristics are displayed in Table 2. The pain-free distance during the 6-min walk test was negatively correlated with HOMA-IR ($p = 0.007$), dyslipidemia ($p < 0.001$), obesity ($p = 0.009$), coronary artery disease ($p = 0.039$), cerebrovascular disease ($p = 0.016$) and chronic kidney disease ($p = 0.012$), whereas the pain-free distance was positively correlated with height ($p = 0.048$), ABI ($p = 0.010$) and male sex ($p = 0.001$). The total distance walked during the 6-min walk test was negatively associated with body mass index ($p = 0.005$), HOMA-IR ($p = 0.014$), hsCRP ($p < 0.001$), dyslipidemia ($p = 0.002$), diabetes ($p < 0.001$), metabolic syndrome ($p = 0.032$), obesity ($p < 0.001$), coronary artery disease ($p = 0.003$), chronic kidney disease ($p = 0.005$) and chronic obstructive pulmonary disease ($p = 0.037$), whereas the total distance walked was positively associated with height ($p < 0.001$), ABI, ($p < 0.001$), HORAC ($p = 0.020$) and male sex ($p < 0.001$).

Multivariate regression models to predict 6-min walk distances are shown in Table 3. Pain-free distance during the 6-min walk test was negatively associated with dyslipidemia ($p < 0.001$), chronic kidney disease ($p = 0.004$), and NF-κB ($p = 0.007$), and was positively associated with height ($p = 0.008$). Total distance walked during the 6-min walk test was negatively associated with NF-κB ($p < 0.001$), coronary artery disease ($p = 0.009$), and BMI ($p = 0.022$), and was positively associated with ABI ($p < 0.001$), male sex ($p < 0.001$), and HORAC ($p < 0.001$).

DISCUSSION

The primary novel finding was that 6-min walk distance in symptomatic patients with PAD was independently and positively associated with circulating antioxidant capacity, and was negatively associated with cultured endothelial cell inflammation, greater PAD severity, coronary artery disease, BMI and female sex. Furthermore, 6-min walk pain-free distance was independently and negatively associated with cultured endothelial cell inflammation, dyslipidemia, chronic kidney disease and shorter height.

Negative Association between Cultured Endothelial Cell NF-κB Activity and 6-min Walk Performance

This is the first study to report that the cultured endothelial cell NF-κB activity was negatively associated with both the total distance and the pain-free distance walked during the 6-min walk test in symptomatic patients with PAD, even after adjusting for ABI and other comorbid conditions. This finding suggests that greater cultured endothelial cell inflammation is associated with worse submaximal exercise performance during a walking task that might be typically encountered in the community setting. These results support our previous study that found cultured endothelial cell NF-κB activity was negatively associated with the claudication onset time (COT) and peak walking time (PWT) during a standardized maximal treadmill test in symptomatic patients with PAD, and with faster oxygen desaturation of the calf musculature during exercise.[23] Collectively, these findings indicate that impairment in the microvasculature is associated with worse performance in both submaximal and maximal exercise tests. It is also noteworthy that cultured endothelial cell NF-κB activity was related to claudication symptoms during both tests, as measured by the pain-free distance during the 6-min walk test in this study and by COT during the treadmill test in our previous study,[23] suggesting that impairment in the microvasculature has an impact on claudication. A potential explanation for heightened cultured endothelial cell NF-κB activity negatively influencing ambulation could be a result of increased inflammatory cytokine expression in PAD patients,[28–30] although we have found that cytokines such as interleukin-6 and tumor necrosis factor alpha are not predictive of gait characteristics in symptomatic patients with PAD.[35]

Positive Association Between Circulating Antioxidant Capacity and 6-min Walk Distance

This is the first study to demonstrate that higher circulating HORAC levels were associated with longer 6-min walk distance in symptomatic patients with PAD. It should be noted that this association existed even after adjusting for ABI, cultured endothelial cell NF-κB activity, sex, body mass index, and presence of coronary artery disease. Consequently, higher circulating antioxidant capacity was associated with better submaximal exercise performance during overground walking independent of severity of PAD, cultured endothelial cell inflammation, and comorbid and demographic measures that include body fatness, presence of coronary artery disease and sex. These results support our previous study that found higher circulating HORAC was positively associated with maximal exercise performance in symptomatic patients with PAD, measured by higher PWT during a standardized maximal treadmill test, and with slower oxygen desaturation of the calf musculature during exercise.[23] The clinical implication of the current findings is that

interventions designed to improve HORAC may improve submaximal exercise performance, as measured by an increase in 6-min walk distance. Circulating antioxidant capacity might be increased through intake of sources of antioxidants in vitamin supplements or in dietary intake of fruits, vegetables, and fiber. We have observed that patients with PAD have relatively poor diets consisting of low intakes of fruits and vegetables, and low intake of fiber.[49] There is a relationship between increased fiber intake and lower hsCRP,[50] suggesting that greater fruit and vegetable consumption may enhance HORAC, thereby lowering inflammation and improving microvascular function.

Comorbid and Demographic Conditions Associated with 6-min Walk Performance

Although the aim of this study was to determine whether circulating biomarkers and cultured endothelial cell measures were associated with 6-min walk performance, we were most interested in whether these associations existed after adjusting for significant covariates. We found that dyslipidemia and chronic kidney disease were negatively associated with pain-free distance, whereas height was a positive predictor of pain-free distance. Additionally, coronary artery disease and body mass index were negatively associated with 6-min walk distance, whereas ABI and male sex were positive predictors of 6-min walk distance.

Our finding that severity of PAD, as measured by ABI, is associated with 6-min walk distance supports previous findings,[17,28] and demonstrates that walking endurance is impaired with lower ABI values (i.e. greater PAD severity). Chronic kidney disease and coronary artery disease were significant negative covariates of pain-free and 6-min walk distance, respectively, suggesting that overt vascular disease in vascular beds other than the lower extremities may be an indicator of greater overall systemic vascular burden, thereby limiting 6-min walk performance. Our current finding that dyslipidemia is negatively associated with pain-free distance supports our previous observation that metabolic syndrome also has a negative impact on pain-free and 6-min walk distance,[51] as dyslipidemia is a component of metabolic syndrome. Nearly all of the patients with dyslipidemia in this study were treated with statins, which improves walking distances.[52] However, there may be some statin-associated myopathy which limits exercise performance such that patients treated with statins still do not perform as well on the 6-min walk test as those patients who do not have dyslipidemia. Body mass index was another covariate negatively associated with 6-min walk distance, which agrees with earlier work.[28] Finally, shorter height and female sex were significantly associated with lower pain-free and 6-min walk distances, respectively, and both may serve as surrogate markers of leg length. Shorter patients and women may take shorter strides during the 6-min walk test, which may decrease the efficiency of performing the test by taking more steps.

Limitations

Study participation may have had a self-selection bias, as patients were volunteers and may represent those with the highest interest in participating, the best access to transportation, and the best health compared to non-volunteers. Additionally, these results are only applicable to PAD patients with claudication, and may not generalize to patients with different disease severity. Another limitation is that circulating oxidative stress biomarkers

were not measured, but may have provided unique information from inflammatory biomarkers.[53] Finally, the cross-sectional design of this study is a limitation because significant associations found among variables does not provide evidence of causality. Although pathophysiological mechanisms cannot be determined from this study, it can be speculated that higher antioxidant capacity, presumably from intake of vitamins A, C, D, and E could have led to vascular alterations such as improvements in endothelial function, inflammation, oxidative stress, and the renin-angiotensin-aldosterone system.[54] These vascular improvements could potentially lead to greater 6-min walk distances in symptomatic patients with PAD. Additionally, the negative association between BMI and 6-min walk distance may have resulted from increased arterial stiffness due to insulin resistance,[55] as well as from greater exertional effort. Although this study was not designed to address these potential mechanisms, our results are generalizable to symptomatic patients with PAD who typically have high prevalence of comorbid conditions.

Conclusion and Clinical Significance

6-min walk performance in symptomatic patients with PAD was associated with vascular biomarkers, as walking distances were negatively associated with cultured endothelial cell inflammation and positively associated with circulating antioxidant capacity. The clinical implication is that behavioral interventions designed to alleviate endothelial cell inflammation and increase circulating antioxidant capacity, such as exercise and antioxidant intake, may improve ambulation of patients with PAD during submaximal exercise that is typically performed during daily activities.

Acknowledgments

Supported by grants from the National Institute on Aging (R01-AG-24296 to AWG; R01-AG047879; R01-AG038747; 3P30AG050911-02S1to AC), the National Center for Complementary and Alternative Medicine (R01-AT006526 to ZU), the National Institute of Neurological Disorders and Stroke (NINDS; R01-NS056218 to AC), the Oklahoma Center for the Advancement of Science and Technology (to AWG, AC, and ZU), and General Clinical Research Center (M01-RR-14467), the American Heart Association (to AC and ZU), and the National Center for Complementary and Alternative Medicine (R01-AT006526 to ZU). Statistical analysis was supported by funding provided by National Institutes of Health, National Institute of General Medical Sciences, grant 1 U54GM104938

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Table 1

Clinical characteristics of 251 symptomatic patients with peripheral artery disease. Values are means (SD) and percentages.

Variables	Values
Age (years)	65 (10)
Weight (kg)	83.9 (18.4)
Height (cm)	168.2 (9.2)
Body Mass Index (kg/m^2)	29.7 (6.2)
Homeostasis Model Assessment-Insulin Resistance	3.3 (5.2)
Ankle-Brachial Index	0.7 (0.2)
6-min Walk Pain-Free Distance (m)	184 (118)
6-min Walk Distance (m)	347 (95)
Hydroxyl Radical Antioxidant Capacity (AU)	1.0 (0.2)
High sensitivity C-Reactive Protein (mg/L)	5.4 (5.9)
Cultured Endothelial Cell Apoptosis (AU)	1.0 (0.4)
Cultured Endothelial Cell Reactive Oxygen Species production (AU)	30.1 (11.3)
Cultured Endothelial Cell NF-κB activity (AU)	1.3 (0.7)
Sex (% Men)	49
Race (% Caucasian)	52
Current Smoking (% yes)	37
Hypertension (% yes)	88
Dyslipidemia (% yes)	90
Diabetes (% yes)	43
Metabolic Syndrome (% yes)	81
Obesity (% yes)	46
Lower Extremity Revascularization (% yes)	35
Coronary Artery Disease (% yes)	34
Cerebrovascular Disease (% yes)	16
Chronic Kidney Disease (% yes)	24
Chronic Obstructive Pulmonary Disease (% yes)	26

Table 2

Associations among 6-min walk distances and clinical characteristics in 251 symptomatic patients with peripheral artery disease.

Variables	6-min Walk Pain-Free Distance	p	6-min Walk Distance	p
Age	0.082	0.198	−0.014	0.828
Weight	−0.065	0.315	−0.011	0.866
Height	0.126	0.048	0.340	<.001
Body Mass Index	−0.111	0.083	−0.178	0.005
Homeostasis Model Assessment-Insulin Resistance	−0.173	0.007	−0.157	0.014
Ankle-Brachial Index	0.165	0.010	0.292	<.001
Hydroxyl Radical Antioxidant Capacity	0.011	0.886	0.184	0.020
High sensitivity C-Reactive Protein	−0.023	0.729	−0.220	<.001
Cultured Endothelial Cell Apoptosis	−0.072	0.361	0.131	0.095
Cultured Endothelial Cell	−0.068	0.404	−0.019	0.821
Reactive Oxygen Species production				
Cultured Endothelial Cell NF-κB activity	−0.151	0.061	−0.120	0.136
Sex (Men)	0.202	0.001	0.368	<.001
Race (Caucasian)	−0.016	0.803	0.036	0.579
Current Smoking	−0.005	0.941	−0.070	0.281
Hypertension	−0.059	0.355	−0.017	0.796
Dyslipidemia	−0.211	<.001	−0.198	0.002
Diabetes	−0.025	0.692	−0.276	<.001
Metabolic Syndrome	−0.102	0.111	−0.137	0.032
Obesity	−0.167	0.009	−0.209	0.001
Lower Extremity Revascularization	−0.082	0.200	−0.114	0.075
Coronary Artery Disease	−0.132	0.039	−0.191	0.003
Cerebrovascular Disease	−0.154	0.016	−0.113	0.077
Chronic Kidney Disease	−0.161	0.012	−0.179	0.005
Chronic Obstructive Pulmonary Disease	0.045	0.482	−0.133	0.037

Values are Pearson correlation coefficients for continuous variables and Spearman correlation coefficients for discrete variables.

Table 3

Regression coefficient summary for predictor variables included in multivariate regression models for 6-min walk distances in 251 symptomatic patients with peripheral artery disease.

Dependent Variables	Predictors	Regression Coefficient	95% Confidence Interval	Partial R^2	P Value
6-min Walk Distance					
	Ankle-Brachial Index	133.14	78.75 to 187.52	0.13	<.001
	Sex (Men)	62.90	36.37 to 89.42	0.09	<.001
	Hydroxyl Radical Antioxidant Capacity	136.68	69.75 to 203.62	0.06	<.001
	Cultured Endothelial Cell NF-κB	−36.93	−56.98 to −16.88	0.04	<.001
	Coronary Artery Disease	−37.59	−65.48 to −9.69	0.03	0.009
	Body Mass Index	−2.70	−5.01 to −0.39	0.01	0.022
	Intercept	232.34	134.23 to 330.46		<.001
6-min Walk Pain-Free Distance					
	Dyslipidemia	−121.21	−187.6 to −54.83	0.07	<.001
	Chronic Kidney Disease	−63.79	−106.94 to −20.64	0.05	0.004
	Cultured Endothelial Cell NF-κB	−36.47	−62.72 to −10.21	0.03	0.007
	Height	2.63	0.70 to 4.55	0.02	0.008
	Intercept	−98.17	−427.89 to 231.56		0.557

Overall model results: 6-min Walk Distance: $R^2 = 0.363$, $p < 0.001$

6-min Walk Pain-Free Distance: $R^2 = 0.167$, $p < 0.001$