Article title: “Impact of exercise on expression of Hsp70 in individuals at risk of peripheral arterial disease”

Authors:

Clara L Perez-Quiroga. MSc UPAEP University, ISSSTEP Specialties Hospital

Javier Rubalcaba-Priego. MD ISSSTEP Specialties Hospital

Jose I Diego Perez-Montes. MD UPAEP University

Virginia Sedeno-Monge. PhD UPAEP University.

Short title: “Exercise on expression of HSP 70 in arterial risk”

PALABRAS:-----

Correspondence author:

Clara Luz Pérez Quiroga. Address: Street 21 sur #1103, Santiago neighborhood. CP 72410 Puebla, Mexico. E-mail: claraluz.perez@upaep.mx

Abstract

- **Objetives** To determine the level of serum expression of the HSP70 protein in individuals at risk of atherosclerotic peripheral arterial disease, after an intervention of moderate exercise.
- **Design** randOmized, single blinded, clinical trial.
- **Setting** Hospital de Especialidades "5 de Mayo" ISSSTEP in Puebla, Puebla, México.
- **Participants** 260 individuals were evaluated by a screening test employing the Ankle-Arm Index (ABI), 220 of them were not within risk value (0.91 to 0.99 mmHg). In the clinical trial, 40 individuals were SELECCIONADOS between February to September of 2019.
- **Interventions** The experimental group started an exercise program to achieve 65% to 80% of the maximum heart rate for 40 minutes by using a pulsometer that measured the heart rate in order to get to the moderate intensity activity goal, the control group received written recommendations to exercise three times per week.
- **Main outcome measures** The lowest of the protein level were in the experimental group, with significant changes, confirming the results of studies in PAD. The highest serum levels of the Hsp70 were found in the group that only received written recommendations (control group).
- **Results** 32 individuals completed the 12-week follow-up. Both the control group (P= 0.004) and the experimental group (P= 0.005) had significant changes in serum levels of the protein (95% Confidence Interval). The risk analysis for PAD using the ABI only showed a significant difference (P= 0.001) in the experimental group (95% Confidence Interval).
- **Conclusions** The risk for PAD can be modified after the intervention of a moderate intensity exercise program and is closely correlated with lower levels of Hsp70 expression.

Keywords: ***Hsp70, aerobic exercise, peripheral arterial disease***

Introduction

The objective of the study is to determine the level of serum expression of the HSP70 protein in individuals at risk of atherosclerotic peripheral arterial disease, after an intervention of moderate exercise.

Atherosclerotic Peripheral Arterial Disease (PAD) is a systemic disease that causes obstruction of arterial blood flow and is a significant cause of morbidity and mortality worldwide [1].

Patients with PAD have a three times greater risk of myocardial infarction, stroke and death than those without the disease. However, more than 50% of patients are asymptomatic; so the disease remains underdiagnosed. [2]

It is estimated that 202 million people in the world are affected with PAD, 45 million of them die from coronary or cerebrovascular disease in a range of ten years [3, 4]

The disease is strongly related to modifiable risk factors (blood pressure, sedentary lifestyle, baseline glycemia, cholesterol, and obesity) and unhealthy lifestyle (tobacco, physical inactivity, food and psychosocial stress), which increases the risk of acquiring the disease up to 17.2 times [4, 5, 6]

Increased membrane permeability leads to the accumulation and modification of proteins, lipids and lipoproteins in the endothelium. The accumulation of pro-inflammatory molecules such as chemo-tactical monocyte protein 1 (MCP-1), intracellular adhesion molecules (ICAM-1), vascular cell adhesion (VCAM-1) and higher nitro-tyrosine content are also generated. [7]

Macrophages are active in response to the spread of the inflammatory response and bind to low-density lipoprotein (LDL) receptors that had internalized.

Macrophages secrete pro-inflammatory cytokines resulting in the recruitment and activation of additional immune cells at the site of the lesion, amplifying the immune response and promoting the development of complex and advanced plaques that become mature atherosclerotic plaques.[8]

The Ankle Arm Index (ABI) is the non-invasive gold standard accepted for both the diagnosis and evaluation of the severity of this disease. The cut-off point for the diagnosis of PAD is an ABI of less than 0.90 at rest, regardless of the Framingham Risk Score, being 95% sensitive in the detection of PAD. [9, 10]

To maintain homeostasis on the vessel wall, vascular cells produce a high level of heat shock proteins (HSP) [11]. These types of stress proteins are cytoprotective agents that promote cell survival during stressors. [12] The reduction of HSP intracellular expression is also related to PAD, cardiovascular diseases and metabolic syndrome, where they are greatly diminished. [8]

HSP are normally intracellular proteins, but when released at an extracellular level, they exert an immune response.[13]

The most thermo-sensitive and highly inducible HSP belongs to the 70 kDa family, as established by Mizzen et al., In 1998 (figure 1). It is believed that extracellular Hsp70 (eHsp70) stimulates innate immunity and acts as a danger signal. Serum Hsp70 has been detected in the peripheral circulation of apparently healthy individuals and increases in response to different stressors, including exercise. [12, 14]

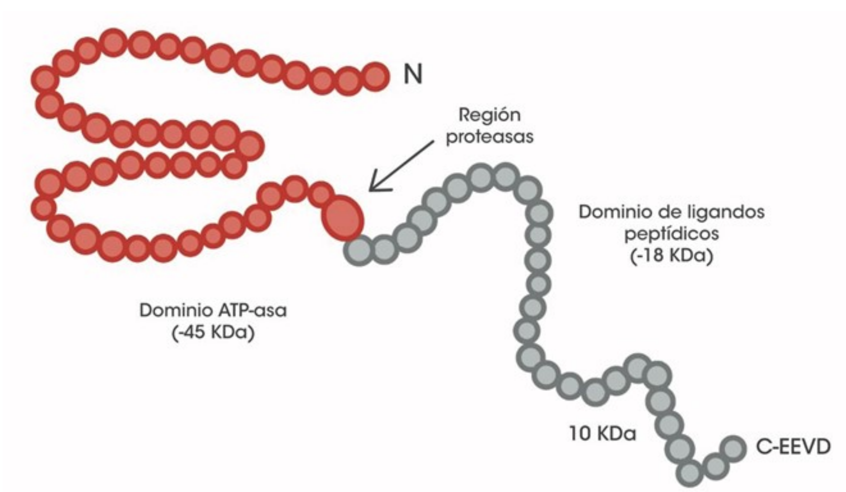

Figure 1. Schematic representation of the molecular structure of HSP70. Adapted from Liu Y et al. [15]

Exercise increases HSP levels primarily through reactive oxygen species (ROS), elevated temperature, hormones, calcium fluxes or mechanical tissue deformation.[16]

It has been shown that the concentration of Hsp70 increases after performing both acute and long-term exercises, reaching levels that remain high, but once its protective action decreases both at intra and extra-cellular levels.[17]

Exercise exerts beneficial effects against atherosclerosis by increasing circulating endostatin, which inhibits the development of atherosclerotic plaque by blocking angiogenesis in plaque tissue.[18]

Isotonic contractions of the type of resistance exercise (aerobic) tend to lead to increases in Hsp70 levels, as opposed to eccentric exercise. With repetitive exercise, an induced increase in this protein is maintained, while the initial response of other HSPs to exercise decreases as training progresses.[16]

Regular exercise initiates long-term processes of adaptation of muscle metabolism, cardiovascular system and immuno-modulatory effects that are largely considered beneficial; There is evidence that during exercise, Hsp70 is released into human circulation in association with exosomes, specifically with aerobic exercise.[19, 20]

It has been shown that Hsp70 directly inhibits inflammatory processes through the suppression of oxidative stress, directly reduces apoptosis, hyperplasia, as well as decreasing the expression of adhesion molecules that lead to leukocyte extravasation and the manifestation of inflammatory cytokines.[16]

Higher serum levels of Hsp70 are associated with reduced thickening of the atherosclerotic intima and a lower risk of coronary artery disease.[21]

Méthods

Study design: This is a randomized clinical trial, single-blind.

Individuals who attended the internal medicine service of the ISSSTEP hospital were invited to participate voluntarily, from February to September of 2019. In order to detect people at risk of PAD, the measurement of the ABI was done. The measure was taken after 10 minutes of resting. The measure was taken in the four extremities to obtain systolic BP of the posterior tibial artery and brachial artery. The cut point to enter the study was between 0.91 and 0.99 mmHg. The informed consent was signed by all the individuals.

The sample selection was done randomized for convenience. A total of 260 candidates were evaluated, 40 of them were selected randomly divided in two by using version 4.2 of EPIDAT, an experimental and a control group. During the progress of the clinical trial four people of each group decided to leave the study by personal decision, thus the groups ended with 16 people each. Dietetic recommendations of low sodium foods were provided to all.

The experimental group started an exercise program to achieve 65% to 80% of the maximum heart rate for 40 minutes by using a pulsometer that measured the heart rate in order to get to the moderate intensity activity goal. Each exercise session included a warm-up and a cool-down phase of 10 minutes at 50 - 60% of the maximum heart rate. The exercise session was 60 minutes long, three times per week, completing 180 minutes per week of moderate intensity exercise, for 12 weeks in total. The control group received written recommendations to exercise three times per week, for a period of 12 weeks but they did not attend the training sessions.

There were two examinations, one at the beginning and the second at the end of the study. Different physiotherapists did the initial and final evaluation. The physiotherapists stayed blind to the group assignation. It was indicated to the participants to not reveal their assignations.

These examinations included anthropometric measurements, including: height, weight, BMI, waist circumference, Systolic BP, Diastolic BP, RHR and the sit and reach test.

Weight, Height and BMI. A weighing machine (seca 700, seca gmbh & co, Hamburg, Germany) was used to measure weight and height. Body Mass Index (BMI) is a simple index that compares weight to height and it is commonly used to classify underweight, overweight and obesity in adults. It is defined as the weight in kilograms divided by the square of the height in meters (kg/m^2). The result of this division is classified as follow: Underweight <18.5 kg/m^2 , Normal range 18.5 – 24.99 kg/m^2 , Overweight ≥25.0 kg/m^2, Preobese 25.0 – 29.99 kg/m^2 , Obese class I 30.0 – 34.99 kg/m^2, Obese class II 35.00 – 39.99 kg/m^2, Obese class III ≥40.00 kg/m^2.

According to the classification, the risk comorbidities are: Low for underweight, average for normal weight, increased for preobese, moderate for obese class I, Severe for obese class II and very severe for obese class III. [22]

Waist Circumference (WC). The WC is a convenient and simple measurement that is unrelated to height. It is measured at the midpoint between the lower border of the rib cage and the iliac crest. To measure the waist, an anthropometric type Rosscraft TAQ tape was used (Rosscraft Innovations, Canada). It classifies people in two groups based on the risk of metabolic complications: Increased in >94 cm in men and >80 cm in women, Substantially increased >102 cm in men and >88 cm in women. [23, 24]

Waist to Height Ratio (WHtR). It is the quotient between WC and height. A WHtR higher than 0.50 is considered to be an indicator of central obesity in adults.

Ankle-Brachial index (ABI). The ABI is an estimation of the prevalence of Peripheral Arterial Disease (PAD). It involves measuring the systolic blood pressure (Systolic BP) in the ankles (dorsalis pedis and posterior tibial arteries) and arms (brachial artery) and then calculating a ratio. The diagnostic criteria for PAD based on the ABI are interpreted as follows: Normal if 0.91–1.30, Mild obstruction if 0.70–0.90, Moderate obstruction if 0.40–0.69, Severe obstruction if <0.40, Poorly compressible if 1.30 An ABI value 1.3 suggests poorly compressible arteries at the ankle level due to the presence of medial arterial calcification.[25]

Systolic and Diastolic Blood Pressure. Blood Pressure (BP) category is defined according to seated clinic BP and by the highest level of BP, whether systolic or diastolic, a blood pressure monitor (ELITE HEM-7130, © OMRON, Japan) was used to measure the systolic BP and the diastolic BP and it is classified as follow for systolic values: Optimal <120 mmHg, Normal 120-129 mmHg, High normal 130-139 mmHg, Grade 1 hypertension 140-159 mmHg, Grade 2 hypertension 160-179 mmhg, Grade 3 hypertension ≥180 mmHg, Isolated systolic hypertension ≥140 mmHg and <90 mmHg in Diastolic Blood Pressure. The Diastolic Blood Pressure (Diastolic BP) is classified as follow: Optimal <80 mmHg, Normal 80-84 mmHg, High normal 85-89 mmHg, Grade 1 hypertension 90-99 mmHg, Grade 2 hypertension 100-109 mmhg, Grade 3 hypertension ≥110 mmHg.[26]

Resting Heart Rate (RHR). It is the lowest heart rate to provide the minimum amount of blood for the body to use while a person is not exercising, the blood pressure monitor (ELITE HEM-7130, © OMRON, Japan) was also used to measure the RHR. According to the American Heart Association (AHA) the normal heart rate is between 60 beats per minute to 100 beats per minute.[27]

The Sit and Reach test. Is used to measure the hip joint angle or sacrum angle in the maximum flexion position while the patients are sit and asked to reach the toes with their fingers, always keeping the ankle in a 90° angle.(LhFx). The values for reference are: Normal ≥2 cm, shortening Grade -3 a -9 cm and shortening Grade II ≤10 cm. [28]

Biomarkers. A commercial kit was used to measure the HSP70 in blood (HSP70 ELISA kit, ©2020 Enzo Life Sciences, Inc., New York, USA) and a professional software was used to read the ELISA by spectrometry with an optic density of 570 ηm.

Analysis of data: All descriptive statistics of the variables in the study were reported as medians, with ± the standard deviation. The non-parametric Mann-Whitney test was used to determine the importance of the difference in the medians between the control group and the experimental group before and after exercise. The differences are considered statistically significant at p values <0.05 with a 95% CI.

RESULTS

Study sample. There were 260 individuals analized, 40 of them were included and 32 were included in the study. Two groups were made, one for control and the other for experimental. Four patients left each group the study because of personal decisions thus both groups ended with 16 patients each (fig 1).

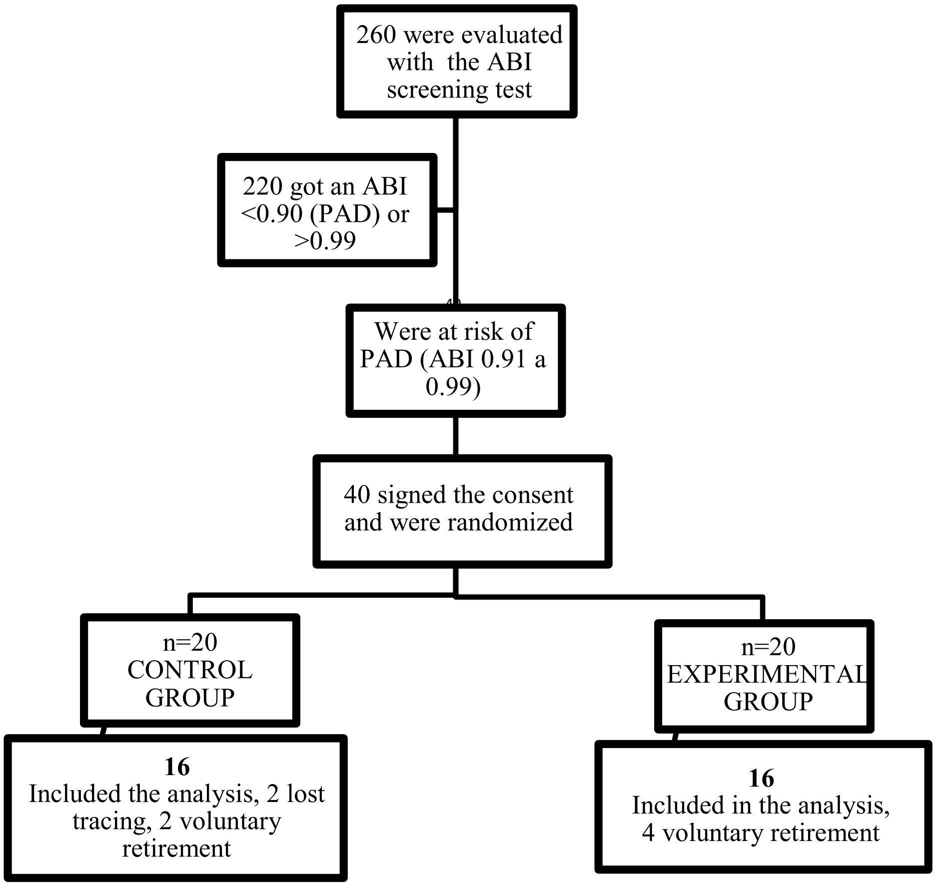

Figure 1. Progress of the participants along the study.
Ankle-Brachial Index: ABI, Peripheral Artery Disease: PAD

Anthropometric effects of exercise training. Cardiovascular medication was not used in either control and experimental groups. During the follow up, minor modifications were noted on anthropometric parameters (Table 1).

Table 1. Comparison between sample and randomised groups at the beginning and at the end of the study. Values are numbers (percentages).

INITIAL RESULTS			
VARIABLES	**SAMPLE**	**CONTROL**	**EXPERIMENTAL**

	(n= 32)	(n=16)	(n=16)
Average age (years)	52.1 (8.5)	53.3 (9.2)	50.9 (7.8)
Normal weight	5 (16%)	5 (31%)	0
Overweight/ obesity	27 (84%)	11 (69%)	16 (100%)
No cardiovascular risk (according to BMI)	6 (19%)	5 (31%)	1 (6%)
Presence of cardiovascular risk (according to BMI)	26 (81%)	11 (69%)	15 (9.4%)
No risk of cardiovascular complications (according to WC)	9 (28%)	6 (38%)	3 (19%)
Presence of risk of cardiovascular complications (according to WC)	23 (72%)	10 (62%)	13 (81%)
No risk of arteriopathy	0	0	0
Presence of arteriopathy risk	32 (100%)	16 (100%)	16 (100%)
Normal BP	27 (84%)	13 (81%)	14 (88%)
Hypertension	5 (16%)	3 (19%)	2 (12%)
Diabetes tipo 2	0	0	0
FINAL RESULTS			
Normal weight	6 (19%)	6 (38%)	0
Overweight/ obesity	26 (81%)	10 (62%)	16 (100%)
No cardiovascular risk (according to BMI)	6 (19%)	6 (38%)	0
Presence of cardiovascular risk (according to BMI)	26 (81%)	10 (62%)	16 (100%)
No risk of cardiovascular complications (according to WC)	8 (25%)	5 (31%)	3 (19%)
Presence of risk of cardiovascular complications (according to WC)	24 (75)	11 (69%)	13 (81%)
No risk of arteriopathy	19 (59%)	9 (56%)	10 (62%)
Presence of arteriopathy risk	13 (41%)	7 (44%)	6 (38%)
Normal BP	27 (84%)	13 (81%)	14 (88%)

Hypertension	3 (9%)	2 (13%)	1 (6%)
Hypotension	2 (6%)	1 (6%)	1 (6%)

DISCUSSION

The relevant finding of this study is the significant decrease in the levels of the Heat Shock Protein Hsp70 in serum, specifically in the group where the exercise was directed, unlike the strategy of written recommendations (control group) after 12 weeks follow-up, although both groups had significant differences. Hsp70 has been recognized to be present in the peripheral circulation of normal individuals, providing the first evidence that Hsp70 can be released into the extracellular environment not only in response to stress but also under physiological conditions. 18, 29 The activation response of Hsp70 depends on the severity and type of stress to which the subject is exposed. 30. In addition to the fact that Hsp70 can modulate vascular contractility 16. Since 2000 31, it has been determined that protein increases in untrained individuals after moderate aerobic exercise, probably as an adaptation of the training load 11, 30, 32. But there are also studies that report its decrease as training progresses 8, 11, 16. In the present study, the application of a training program from 65 to 80% of the maximum heart rate, to achieve the adaptation of cumulative episodes and the recovery of the physiological stressor as the training progressed in the experimental group. The application of exercise with moderate intensity for 12 weeks was sufficient to restore and decrease the expression of the Hsp70.

The family of 70 kDa Heat Shock Proteins (HSP70) has been widely studied for its participation in obesity, inflammation and endothelial dysfunction 33, 34. Since 2000 Wright et al.35 have shown that serum levels of Hsp70 increases in patients with PAD, various epidemiological studies have confirmed that serum levels of the protein Hsp70 are elevated in peripheral arterial disease 36, in more severe arterial calcification and continue to increase in patients with peripheral atherosclerosis 34 and the presence of an antiatherogenic status in the vasculature 33, 37. In our study group, the highest serum levels of the Hsp70 were found in the group that only received written recommendations (control group). The lowest of the protein level were in the experimental group, with significant changes, confirming the results of studies in PAD.

Peripheral arterial disease (PAD) increases its prevalence with age and is associated with a reduction in life expectancy 38. The presence of PAD has been shown to be a marker of cardiovascular disease 39 and is little recognized in primary care practices 40. The present study examined patients at risk of PAD in a hospital setting, using the oscillometric arm-ankle index (ABI), which indicates the degree of risk for PAD. The oscillometric method is easy, simple, requires minimal training, and can be used in large and small hospitals, health centers, or even at home 39, 41. Transient responses to stress induced by regular and moderate exercise tend to down regulate vascular inflammation and protect vessels from injury 9, 16. Peripheral resistance decreases during exercise, which increases blood flow to peripheral musculature 40. Significant changes were found in the experimental group, with an average of end of study out of risk level for PAD; however, there were no significant changes in the group of written recommendations, maintaining an average risk for the disease, statistically verified. The ABI is the accepted non-invasive gold standard both for the diagnosis of PAD and for the assessment of the severity of the disease 41, it has been described as a strong predictor of mortality in patients with PAD 38. The measurement of the ABI should be performed routinely in patients with intermediate / high risk, allowing early intervention and monitoring of populations at risk, 1, 2. Coinciding with the results of our study.

Classic risk factors for the development of PAD, such as diabetes 4, 5 were not included in our study, where no patient had this diagnosis; However, the risk factors for PAD considered in this investigation were the level of blood pressure (both systolic and diastolic), the waist circumference, the waist-height index; as well as the body mass index, respectively. There were no significant changes at the beginning and end of the study in any of the groups. The level of the baseline heart rate contemplated in the present investigation had a significant change in the experimental group, compared to the control group. Higher resting HR has been shown to correlate with an increased risk of cardiovascular disease and death in the general population. The heart rate response to stress, exercising or drug injection, has been shown to have prognostic and diagnostic power 27. Regular exercise appears to be inversely related to life expectancy 42 and positively related to cardiovascular and all-cause mortality 43, 44. There are intervention studies on physical training, however, to date, according to the evidence sought, we have not found clinical trials for PAD that examine these clinical levels and their association with physical exercise.

Lower body flexibility was tested by attempting to reach distance from the subject's fingertips and toes with the legs extended. Poor trunk flexibility could be associated with higher arterial stiffness in middle-aged and older people, regardless of other fitness components 45, 46. Therefore, poor flexibility may be a useful indicator of physical fitness for remodeling arterial 47, as studies reveal. In our study there were no significant changes after the implementation of an exercise program, this may be due in part to the objective of the study, it was found a potential role of moderate intensity exercises to improve the risk of PAD, having an improvement in terms of the decrease in distance, but not significantly in the experimental group.

LIMITATIONS

The characteristics of the subjects in this study limited the generalizability of the results. All the participants belonged to the hospital's medical service, middle-aged Mexicans, and our findings cannot beapplied to subjects of other ages or ethnicities. There were only two times for the application of the tests, at the beginning and at the end of the study, which limits the results. The number of participants for the study was small, considering the subjects previously evaluated for participation in the study. However, this is the first investigation related to the risk of PAD, exercise, and serum Hsp70 levels. More research with different degrees of the disease is needed to confirm the new findings.

CONCLUSION

This is the first study in humans to investigate the serum level of the Hsp70 protein in association with the risk of PAD, after the implementation of a moderate exercise program. Our findings indicate that the risk for PAD changes after the intervention of the exercise program and is closely correlated with lower levels of Hsp70 expression. More prospective studies are required to establish the possible biomarker role of

Hsp70 in PAD, in order to clarify whether elevated circulating levels of Hsp70 are a consequence of having an increased risk of PAD; as well as the role of moderate exercise in the prevention.

References

1. Urbano L, Portilla E, Muñoz W, Hofman A, Sierra-Torres. Prevalence and risk factors associated with peripheral arterial disease in an adult population from Colombia. Arch Cardiol Mex. 2018 Apr-Jun; 88(2):107-115. doi: 10.1016/j.acmx.2017.02.002
2. Verberk WJ, Kollias A, Stergiou GS. Automated oscillometric determination of the ankle-brachial index: a systematic review and meta-analysis. Hypertens Res. 2012 Sep;35(9):883-91. doi: 10.1038/hr.2012.83
3. Andras A, Ferket B. Screening for peripheral arterial disease. Cochrane Database of Systematic Reviews 2014; 4 doi: 10.1002/14651858.CD010835.pub2
4. Gerhard-Herman MD, Gornik HL, Barrett C, Barshes NR, Corriere MA, Drachman DE et al. 2016 AHA/ACC Guideline on the Management of Patients With Lower Extremity Peripheral Artery Disease: Executive Summary: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. Circulation. 2017 Mar 21;135(12):e686-e725. doi: 10.1161/CIR.0000000000000470
5. Aboyans V, Ricco JB, Bartelink MEL, Björck M, Brodmann M, Cohnert T et al. 2017 ESC Guidelines on the Diagnosis and Treatment of Peripheral Arterial Diseases, in collaboration with the European Society for Vascular Surgery (ESVS): Document covering atherosclerotic disease of extracranial carotid and vertebral, mesenteric, renal, upper and lower extremity arteriesEndorsed by: the European Stroke Organization (ESO)The Task Force for the Diagnosis and Treatment of Peripheral Arterial Diseases of the European Society of Cardiology (ESC) and of the European Society for Vascular Surgery (ESVS). Eur Heart J. 2018 Mar 1;39(9):763-816. doi: 10.1093/eurheartj/ehx095
6. Shah N, Allison M, Teng Y, Wassertheil-Smoller S, Sotres-Alvarez D, Ramos AR et al. Sleep Apnea is Independently Associated with Peripheral Arterial Disease in the Hispanic Community Health Study/Study of Latinos. Arterioscler Thromb Vasc Biol. 2015 March; 35(3): 710–715. doi: 10.1161/ATVBAHA.114.304625

7. Noble EG, Shen GX. Impact of exercise and metabolic disorders on heat shock proteins and vascular inflammation. Autoimmune Dis. 2012;2012:836519. doi: 10.1155/2012/836519

8. Tiss A, Khadir A, Abubaker J, Abu-Farha M, Al-Khairi I, Cherian P et al. Immunohistochemical profiling of the heat shock response in obese non-diabetic subjects revealed impaired expression of heat shock proteins in the adipose tissue. Lipids in Health and Disease. 2014; 13(106) doi: 10.1186/1476-511X-13-106

9. Castro-Sánchez AM, Moreno-Lorenzo C, Matarán-Peñarrocha GA, Feriche-Fernández-Castanys B, Sánchez Labraca N, Sánchez Joya Mdel M. Efficacy of a massage and exercise programme on the ankle-brachial index and blood pressure in patients with diabetes mellitus type 2 and peripheral arterial disease: a randomized clinical trial]. Med Clin (Barc). 2010 Feb 6;134(3):107-10. Spanish. doi: 10.1016/j.medcli.2009.07.018

10. Casey S, Lanting S, Oldmeadow C, Chuter V. The reliability of the ankle brachial index: a systematic review. J Foot Ankle Res. 2019 Aug 2;12:39. doi:10.1186/s13047-019-0350-1

11. Whitham M, Fortes MB. Heat shock protein 72: release and biological significance during exercise. Front Biosci. 2008 Jan 1;13:1328-39. doi: 10.2741/2765

12. Vardiman JP, Jefferies L, Touchberry C, Gallagher P. Intramuscular heating through fluidotherapy and heat shock protein response. J Athl Train. 2013 May-Jun;48(3):353-61. doi: 10.4085/1062-6050-48.2.22

13. Zininga T, Ramatsui L, Shonhai A. Heat Shock Proteins as Immunomodulants. Molecules. 2018 Nov 1;23(11):2846. doi:10.3390/molecules23112846

14. Périard JD, Ruell P, Caillaud C, Thompson MW. Plasma Hsp72 (HSPA1A) and Hsp27 (HSPB1) expression under heat stress: influence of exercise intensity. Cell Stress Chaperones. 2012 May;17(3):375-83. doi: 10.1007/s12192-011-0313-3

15. Liu Y, Gampert L, Nething K, Steinacker JM. Response and function of skeletal muscle heat shock protein 70. Front Biosci. 2006 Sep 1;11:2802-27. doi: 10.2741/2011

16. Noble EG, Shen GX. Impact of exercise and metabolic disorders on heat shock proteins and vascular inflammation. Autoimmune Dis. 2012;2012:836519. doi: 10.1155/2012/836519

17. Maugeri N, Radhakrishnan J, Knight JC. Genetic determinants of HSP70 gene expression following heat shock. Hum Mol Genet. 2010 Dec 15;19(24):4939-47. doi: 10.1093/hmg/ddq418

18. Schüttler D, Clauss S, Weckbach LT, Brunner S. Molecular Mechanisms of Cardiac Remodeling and Regeneration in Physical Exercise. Cells. 2019 Sep 23;8(10):1128. doi: 10.4061/2011/972807

19. Frühbeis C, Helmig S, Tug S, Simon P, Krämer-Albers EM. Physical exercise induces rapid release of small extracellular vesicles into the circulation. J Extracell Vesicles. 2015 Jul 2;4:28239. doi: 10.3402/jev.v4.28239

20. De Maio A, Vazquez D. Extracellular heat shock proteins: a new location, a new function. Shock. 2013 Oct;40(4):239-46. doi:10.1097/SHK.0b013e3182a185ab

21. Xu Q, Metzler B, Jahangiri M, Mandal K. Molecular chaperones and heat shock proteins in atherosclerosis. Am J Physiol Heart Circ Physiol. 2012 Feb 1;302(3):H506-14. doi: 10.1152/ajpheart.00646.2011

22. Romero-Corral A, Montori VM, Somers VK, Korinek J, Thomas RJ, Allison TG. Association of bodyweight with total mortality and with cardiovascular events in coronary artery disease: a systematic review of cohort studies. Lancet. 2006 Aug 19;368(9536):666-78. doi: 10.1016/S0140-6736(06)69251-9

23. Gullaksen S, Funck KL, Laugesen E, Hansen TK, Dey D, Poulsen PL. Volumes of coronary plaque disease in relation to body mass index, waist circumference, truncal fat mass and epicardial adipose tissue in patients with type 2 diabetes mellitus and controls. Diab Vasc Dis Res. 2019 Jul;16(4):328-336. doi: 10.1177 / 1479164119825761

24. Reis JP, Allen N, Gunderson EP, Lee JM, Lewis CE, Loria CM. Excess body mass index- and waist circumference-years and incident cardiovascular disease: the CARDIA study. Obesity (Silver Spring). 2015 Apr;23(4):879-85. doi: 10.1002 / oby.21023

25. Ashwell M, Gunn P, Gibson S. Waist-to-height ratio is a better screening tool than waist circumference and BMI for adult cardiometabolic risk factors: systematic review and meta-analysis. Obes Rev. 2012 Mar;13(3):275-86. doi: 10.1111/j.1467-789X.2011.00952.x

26. Williams B, Mancia G, Spiering W, Agabiti Rosei E, Azizi M, Burnier M. 2018 ESC/ESH Guidelines for the management of arterial hypertension: The Task Force for the management of arterial hypertension of the European Society of Cardiology and the European Society of Hypertension: The Task Force for the management of arterial hypertension of the European Society of Cardiology and the European Society

of Hypertension. J Hypertens. 2018 Oct;36(10):1953-2041. doi: 10.1097/HJH.0000000000001940

27. Hawkins SM, Guensch DP, Friedrich MG, Vinco G, Nadeshalingham G, White M. Hyperventilation-induced heart rate response as a potential marker for cardiovascular disease. Sci Rep. 2019 Nov 29;9(1):17887. doi: 10.1038/s41598-019-54375-9

28. Ayala F, Sainz de Baranda P, De Ste Croix M, Santonja F. Absolute reliability of five clinical tests for assessing hamstring flexibility in professional futsal players. J Sci Med Sport. 2012 Mar;15(2):142-7. doi: 10.1016/j.jsams.2011.10.002

29. Radons J. The human HSP70 family of chaperones: where do we stand? Cell Stress Chaperones. 2016 May; 21(3):379-404. doi: 10.1007/s12192-016-0676-6

30. Cuthbert RL, Shute RJ, Slivka DR. Skeletal muscle cold shock and heat shock protein mRNA response to aerobic exercise in different environmental temperatures. Temperature (Austin). 2018 Dec 16;6(1):77-84. doi: 10.1080/23328940.2018.1555414

31. Febbraio MA, Koukoulas I. HSP72 gene expression progressively increases in human skeletal muscle during prolonged, exhaustive exercise. J Appl Physiol (1985). 2000 Sep;89(3):1055-60. doi: 10.1152/jappl.2000.89.3.1055

32. Henstridge DC, Febbraio MA, Hargreaves M. Heat shock proteins and exercise adaptations. Our knowledge thus far and the road still ahead. J Appl Physiol (1985). 2016 Mar 15;120(6):683-91. doi: 10.1152/japplphysiol.00811.2015

33. Costa-Beber LC, Hirsch GE, Heck TG, Ludwig MS. Chaperone duality: the role of extracellular and intracellular HSP70 as a biomarker of endothelial dysfunction in the development of atherosclerosis. Arch Physiol Biochem. 2020 Apr 15:1-8. doi: 10.1080/13813455.2020.1745850

34. Rodríguez-Iturbe B, Johnson RJ. Heat shock proteins and cardiovascular disease. Physiol Int. 2018 Mar 1;105(1):19-37. doi: 10.1556/2060.105.2018.1.4

35. Wright BH, Corton JM, El-Nahas AM, Wood RF, Pockley AG. Elevated levels of circulating heat shock protein 70 (Hsp70) in peripheral and renal vascular disease. Heart Vessels. 2000;15(1):18-22. doi: 10.1007/s003800070043

36. Krepuska M, Szeberin Z, Sótonyi P, Sarkadi H, Fehérvári M, Apor A, Rimely E et al. Serum level of soluble Hsp70 is associated with vascular calcification. Cell Stress Chaperones. 2011 May;16(3):257-65. doi: 10.1007/s12192-010-0237-3

37. Xie F, Zhan R, Yan LC, Gong JB, Zhao Y, Ma J, et al. Diet-induced elevation of circulating HSP70 may trigger cell adhesion and promote the development of atherosclerosis in rats. Cell Stress Chaperones. 2016 Sep;21(5):907-14. doi: 10.1007/s12192-016-0716-2

38. Clemens RK, Annema W, Baumann F, Roth-Zetzsche S, Seifert B, von Eckardstein A, et al. Cardiac biomarkers but not measures of vascular atherosclerosis predict mortality in patients with peripheral artery disease. Clin Chim Acta. 2019 Aug;495:215-220. doi: 10.1016/j.cca.2019.04.061

39. Crawford F, Welch K, Andras A, Chappell FM. Ankle brachial index for the diagnosis of lower limb peripheral arterial disease. Cochrane Database Syst Rev. 2016 Sep 14;9(9):CD010680. doi: 10.1002/14651858.CD010680.pub2

40. Alqahtani KM, Bhangoo M, Vaida F, Denenberg JO, Allison MA, Criqui MH. Predictors of Change in the Ankle Brachial Index with Exercise. Eur J Vasc Endovasc Surg. 2018 Mar;55(3):399-404. doi: 10.1016/j.ejvs.2017.12.004

41. Chongthawonsatid S, Dutsadeevettakul S. Validity and reliability of the ankle-brachial index by oscillometric blood pressure and automated ankle-brachial index. J Res Med Sci. 2017 Apr 26;22:44. doi: 10.4103/jrms.JRMS_728_16

42. Ó Hartaigh B, Gill TM, Shah I, Hughes AD, Deanfield JE, Kuh D, Hardy R. Association between resting heart rate across the life course and all-cause mortality: longitudinal findings from the Medical Research Council (MRC) National Survey of Health and Development (NSHD). J Epidemiol Community Health. 2014 Sep;68(9):883-9. doi: 10.1136/jech-2014-203940

43. Aune D, Sen A, ó'Hartaigh B, Janszky I, Romundstad PR, Tonstad S, Vatten LJ. Resting heart rate and the risk of cardiovascular disease, total cancer, and all-cause mortality - A systematic review and dose-response meta-analysis of prospective studies. Nutr Metab Cardiovasc Dis. 2017 Jun;27(6):504-517. doi: 10.1016/j.numecd.2017.04.004

44. Reimers AK, Knapp G, Reimers CD. Effects of Exercise on the Resting Heart Rate: A Systematic Review and Meta-Analysis of Interventional Studies. J Clin Med. 2018 Dec 1;7(12):503. doi: 10.3390/jcm7120503

45. Nishiwaki M, Kurobe K, Kiuchi A, Nakamura T, Matsumoto N. Sex differences in flexibility-arterial stiffness relationship and its application for diagnosis of arterial stiffening: a cross-sectional observational study. PLoS One. 2014 Nov 26;9(11):e113646. doi: 10.1371/journal.pone.0113646

46. Yamamoto K, Kawano H, Gando Y, Iemitsu M, Murakami H, Sanada K. Poor trunk flexibility is associated with arterial stiffening. Am J Physiol Heart Circ Physiol. 2009 Oct;297(4):H1314-8. doi: 10.1152 / ajpheart.00061.2009

47. Suwa M, Imoto T, Kida A, Yokochi T, Iwase M, Kozawa K. Association of body flexibility and carotid atherosclerosis in Japanese middle-aged men: a cross-sectional study. BMJ Open. 2018 Jan 5;8(1):e019370. doi: 10.1136/bmjopen-2017-019370