Association of **protein concentration and enzyme AChE activity** in plasma and whole blood samples with physical activity in healthy elderly individuals

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Abstract

Background:

Physical activity influence protein concentration and AChE level in human blood. The aim of this study was to assess influence of continuous monthly physical activity in healthy elderly individuals.

Methods:

Data analysis was performed on **total 62 participants**. Participants differ based on gender and age. Level of activation and inhibition of enzyme AChE in blood has been measured utilizing substrate acetylcholine chloride (AChCL) and inhibitor eserine 0.1mM. The study has been expanded to investigate efficacy of enzyme AChE inhibition with BW284c51 0.01mM inhibitor.

Data analysis is carried out with Microsoft excel 2007. Statistical software for analysis is IBM SPSS v 23.0. Descriptive statistics (mean +-SD) and parametric statistical tests (Pearson, Linear stepwise regression, T test and One way ANOVA) were assessed before and after 3 months of physical activity.

Results:

Continual monthly physical activity **has** an effect on in vitro protein concentration, activation/inhibition of enzyme AChE in plasma and whole blood samples. Usage of diverse statistical test indicated diverse interpretation of final results.

Conclussion:

Overall outcome confirms that monthly sport activity causes change of protein concentration and enzyme AChE activity in blood samples, making it a good strategic biomarker in health and disease. Physical activity has a beneficial effect on human wellbeing and disease prevention in healthy elderly individuals.

Keywords: physical activity, blood biomarkers, protein concentration, enzyme AChE, elderly individuals, healthy individuals

1. Background:

Cholinesterases are key enzymes involved in various cellular activities. The properties of activation and inhibition of cholinesterase enzymes as biomarkers in humans have long been known for monitoring: organophosphate and carbamate poisoning during food consumption, in professional and environmental medicine for disease diagnosis, therapy and treatment [1]. Cholinergic transmission disorder is a well-established pathological pattern of cancer, neurodegenerative and inflammatory diseases such as: Alzheimer's and Parkinson's disease, Amyotrophic lateral sclerosis, lung, prostate, breast, colon, kidney, liver, head and neck cancer [2-4]

Increased number of human disorder present great socio economic burden worldwide. Based on the WHO statistics, age of disease onset and death significantly varies by country [5]. During the last 15 years, the leading cause of death appertains to cardiovascular disease, causing 15.2 million deaths out of 56.9 million in 2016 year [6]. Cancer as second world wide leading cause of death globally took 9.6 million lives in 2018 [6]. However, based on other statistic, the second most common cause of death in 2016 year is given rise to neurodegenerative disorders, which is not in the accordance to the WHO statistics [6, 7].

Such an increase in prevalence and data variability can be explained by a mixture of different factors such as a combination of genetic and environmental factors, the sensitivity of statistic and detection methods, therapy efficacy, as well as data quality and completeness of the reports [8-10]. Understanding changes that accompany the molecular mechanisms of the disease is complex. Depending on the disease, changes include value variability of clinical parameters in blood and urine, neuro-immune system disorders, altered tissue homeostasis and blood flow, impaired tissue homeostasis and protein modification, accumulation and aggregation [11].

Physical activity as an environmental factor can have a positive or negative effect on organism. Beneficial activity is looked in maintaining body weight, improving lung and heart capacity, strength and endurance of bones and muscles, physical condition [12]. Harmful substances are released from the body, affecting the brain and intellectual capacity, removing stress, slowing aging and improving sleep [12]. Physical activity causes change of physiological processes, leading to an increase in serotonin and inflammatory factors, modulation of neurotransmitters and alleviation of symptoms by lowering blood pressure, triglycerides, LDL and CRP, and increasing HDL levels in the blood [12-14].

However, intense and prolonged physical activity causes stress, free radicals and mediated damage, inflammation, muscle fatigue [12].

Knowledge of all homeostatic/pathological conditions in the body is crucial for finding appropriate **diagnostic and therapeutic biomarkers for the monitoring and treatment** of human diseases [11].

Cholinesterases are neurotransmitters that catalyze the hydrolysis of the cholinergic neurotransmitter acetylcholine, allowing cholinergic neurons to return to a state of rest after activation [1]. The cholinesterase enzyme family is the hydrolase of carboxyl esters. They are present in the central nervous system, especially in the nervous tissue, muscles and blood cells, but also in other tissues [2, 3].

There are two cholesterases that differ in substrate specificity, kinetics and activities in different regions of the brain, Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE). **Butyrylcholinesterase (plasmatic, pseudocholinesterase)** belong to a family of serine protease. BChE gene is located on chromosome 3, and there are no alternative forms of gene splicing. BChE is produced in the liver and circulates in the blood. Whole blood samples are the most prevalent with BChE molecule. Level of BChE enzyme decreases during liver disease, pregnancy and cancer [15].

Acetilcholinesterase (blood, erythrocyte cholinesterase) is located on chromosome 7 (q22.1), where structural/functional diversity arises from alternative splicing of mRNA molecules and posttranslational association of kinetic and structural subunit [2, 16]. The most prevalent form of AChE is located on erythrocyte membrane in plasma. Structural organization, isoforms, enzyme structure, biogenesis, transport, processing and localization, molecular signalization, polymorphisms, frequencies of SNP, and haplotypes along with 3D structure is known for AChE enzyme [2, 3, 16-18]. Moreover, the basis for functional and pharmacogenetic characterization of naturally occurring AChE protein variants is provided [17].

Although cholinergic transmission disorder is a well-established pathological pattern of cancer, neurodegenerative and inflammatory diseases, **treatment** is in the most cases poorly effective/ineffective and acts to reduce symptoms and not to completely cure the disease [2-4, 19]. Based on this notion, it is of great importance to find appropriate diagnostic and therapeutic biomarkers for monitoring and treating diseases. Normally present cholinesterase inhibitors are located in a large number of daily consumed foods such as: potatoes, tomatoes, peppers, goji berries, blueberries, broccoli, carrots [20-22].

The most commonly used synthetic AChE inhibitors for disease treatment are eserin and BW284c51 [23, 24].

Physical activity reduces the risk of cancer, cardiovascular and neurodegenerative diseases and is enforced as a suitable complementary therapy for disease treatment [25, 26]. Increase of AChE enzyme activity is noticeable in healthy male swimmers [27]. Changes of AChE enzyme activity in blood reflect neurodegenerative processes in the brain [28]. Physical activity **reduces AChE activity** and improves cognition and memory in rats [29]. Cholinesterases are involved in tumor genesis. Depending on the cancer type there is increase/decrease in AChE and BChE activity, enhancing cholinergic signaling and tumor progression [30, 31]. Physical activity reduces cancer symptoms and treatment side effects [32].

Very few studies have been conducted to understand the significance of AChE biomarkers in healthy subjects and use the knowledge to compare them with diseased patients. The available

literature contains insufficient information to draw conclusions. There is an urgent need for a simple, fast, economical and reliable biomarker.

Thus, the current study done as a **part of PhD thesis** is an attempt to assess the level of AChE in healthy subjects under the influence of continual 3 months physical activity before conducting a study on diseased patients and giving definitive therapy. The definition of the research task is guided by the fact that the pathogenesis of human diseases can be described by several mechanisms, among which is the significant influence of AChE enzyme as a potential cause of disease, diagnosis, prognosis, therapy and treatment. Due to its characteristic properties the AChE enzyme has become the **main model** for investigating the relationship between clinical markers (anthropometry, blood, urine) and changes in the physiological state of the organism

Based on this information above, the **hypothesis** is set, stating that: the changes in the protein concentration and AChE activity in blood is associated with physical activity duration **in elderly healthy subjects**.

In order to test the working hypothesis, the following research objectives were set to examine the effect of 3 months physical exercise on **protein concentration, AChE enzyme activity, inhibitor efficacy of inhibitor eserin and BW284c51**.

Based on the fact that pathogenesis of the disease can be explained by diverse number of biomarkers, among which is enzyme AChE in blood, we will discuss how exercise can affect **AChE and protein concentration in healthy elderly participants.**

Objective of the paper was to investigate the effect of endurance physical activity before and after three months of physical activity in plasma and whole blood samples in:

- In vitro protein concentration
- In vitro activation of enzyme AChE with substrate acetylcholine chloride (AChCL)
- in vitro inhibition of enzyme AChE with inhibitor (0.1mM eserine)
- how values of blood marker AChE and anthropometry are correlated
- what is the difference in inhibitor efficacy between inhibitor eserine and BW284c51

Main hypothesis being tested is that physical activity influence values of investigated parameters of anthropometry, biomarker level of protein concentration, enzyme AChE in blood and inhibitor efficacy of inhibition. Moreover, this study postulates that:

- Physical activity influence protein concentration
- Physical activity influence basal level of enzyme AChE activation
- Physical activity influence efficacy of enzyme AChE inhibition using inhibitor eserine
- Basal and inhibition level of AChE enzyme has correlation with anthropometry parameters.

2. Material and methods

2.1 Material

2.1.1 Human biological material

Experiments were performed according to **ethical standards** and with written consent of the blood donors. The study was given ethical permission from the National Medical Ethics Committee (number 82/07/14). Experimental part of the PhD thesis was done at the Biotechnical Faculty, University of Ljubljana, Slovenia, within the project **J5-7098** and **CEEPUS free mover PhD student program mobility** with the University of Belgrade, Serbia.

Total number of participants at the beginning of the study was 62. At the end of the study 37 healthy elderly participants completed the survey process. Participants differed based on gender, age and anthropometric measurements. Protein concentration, level of basal AChE enzyme activation/inhibition of AChE with acetilcholinechloride and eserin/bw284c51 is measured. Study evaluated the influence of 3 months physical activity on whole blood and plasma protein concentration and ACHE activity in healthy elderly individuals.

2.2 Methods

2.2.1 Sampling of biological material

Whole blood samples were collected in healthy individuals by venepuncture using needles of 21 diameter (70mm length, 0.4 mm inner diameter, Microlance, Becton Dicinson, Franclin Lakes, NJ, USA) and stored in four 2.7mL vacutainer containing 270 µL trisodium citrate (0.109 mol / L) as anticoagulant.)

Blood elements, plasma and erythrocyte membrane, were isolated from the subject biological material. Protein concentration was assessed on microtiter plate. The measurement of cholinesterase activity in whole blood, plasma, and erythrocyte samples was accomplished using the Elman et all method (1961),

2.2.2 Fractioning of biological material

a) Plasma isolation:

100µL of whole blood was sampled into the ependorf tube to measure cholinesterase activity. In order to separate cells from plasma, blood was centrifuged at 1550 g, 37 ° C, for 20 minutes in a centric 200/R centrifuge (Domel doo, Železniki, Slovenia). After a first centrifugation, the upper plasma fraction was separated in a centrifuge tube (1.5 mL). Plasma was placed in Ependorf tubes. Leukocyte sheath was removed from red blood cells before and after centrifugation.

The red blood cells were washed twice in phosphate-citrate buffer solution (PBS citrate buffer; 137mM NaCL, 2.7 mM KCL, 7.8 mM Na2HPO4 * 2H2O, 1.5 mM KH2PO4, containing 10.9 mM trisodium citrate; pH 7.4) for 5 min. at 1550 g, 37°C; Centric 200/R centrifuges (Domel doo, Železniki, Slovenia).

Note1: all plasma or part of the buffer (supernatant) was removed and replaced with 1 mL of fresh PBS citrate buffer; after the final second centrifugation, the supernatant was removed and the red blood cells were stored in the same vial.

Note2: As a preliminary test, the leukocyte sheath was stored in a 2.7mL vial without the presence of anticoagulants and analyzed without washing or further processing.

For the next experiment: The leukocyte sheath was pipette using 1mL wider extensions, while the polysaccharide gradient was used to isolate the monocytes. The procedure used did not provide a pure white blood cell fraction as the plasma and red blood cells remained in the fraction. The results will not be considered in the analysis.

b) Erythrocyte membrane isolation:

During erythrocyte membrane fractionation, 500 µL of whole blood was collected in 1.5mL ependorf tube using 100 µL extensions (note: it is best to use a larger extension than 1mL during pipetting, in order to avoid the destruction of red blood cells). Centrifuge at 200 g (= 1440 rpm, Železniki Technique, 322A) for 5 min, at 4◦C to remove plasma. About 200 µL of blood plasma was removed and the remaining suspension was washed 3 times with cold 1X PBS citrate buffer (10mM PBS citrate buffer; 137 mM NaCL, 2.7 mM KCl, 7.8 mM Na2HPO4 • 2H2O, 1.5 mM KH2PO4 containing 10.9mM trisodium citrate; pH 7.4). The erythrocytes were resuspended in hypotonic PBS citrate buffer with 5mM Tris HCL containing 1mM EDTA pH 7.4 (about 1000 µL fresh buffer added after washing) and left at 10◦C overnight. The erythrocytes were centrifuged 7-8 times in hypotonic PBS citrate buffer at 9000 rpm for 10 min at 4◦C to obtain erythrocyte membranes (ER spirit) at the base of the ependorf. The erythrocyte membranes thus prepared (after loss of hemoglobin) were treated 3X for 30 sec with Triton X-100 detergent (0.1%, prepared in 25mM phosphate buffer, pH 7.4) to dissolve the membrane-bound enzyme, acetylcholine esterase (AcetylCholin esterase- AChE). The homogenate was centrifuged at 10,000 g for 10 min at 4-6 ◦C and the pure supernatant was used for AChE analysis.

2.2.3 Biochemical methodology

a) <u>Measurement of protein concentration</u>

To measure protein absorption on a microtiter plate, we have 3 types of reaction: sample reaction, negative control reaction, and standard curve (bovine serum albumin-BSA) dilution of 2mg / ml, 1mg / ml, 0.5mg / ml, 0.25mg / ml, 0.125mg / ml, 0.0625 mg / ml). We did triplicates for each reaction. Depending on the type of reaction in each well, we had: 20 µL of sample / buffer / BSA dilution and 200 µL of a mixture of reagent A and reagent B in a ratio of 1:50). The absorbance was measured at **550nm** using a spectrophotometer (BioTek, Cytation 3, Bad Friedrichshall, Germany) for one cycle (30 sec). Based on the absorbance values obtained, the protein concentration of each sample was calculated using a Microsoft excel 2007 program.
Technical view1: To prepare a dilution solution, equation C1=(C2xV2)/V1 is used to calculate stock solution. Upon gaining absorbance values of triplicate, mean value was calculated in

Microsoft Excel 2007. Blank value is subtracted from the average. Standard curve of linear regression with equation is assessed in the form y=aX + b and R2 value, where a is line slope; b is intercept, and R is correlation coefficient. R2 value closer to 1 indicated a good fit of the standard curve and linear regression. Protein concentration [mg/ml] is calculated, taking into the account dilutions for each sample.

b) <u>Measuring cholinesterase activity:</u>

To measure cholinesterase activity on a microtiter plate, we made 3 types of reaction, induced reaction, inhibited reaction, and negative control reaction. We did triplicates for each reaction. Depending on the type of reaction in each well for the endogenous/induced/inhibited reaction, we have 100 µL of diluted sample, 100 µL of Elman reagent (DTNB in 250mM KP buffer, pH 7.4) without/with acetylthiocholine chloride substrate (final concentration of 1mM) and 20 µL of KP buffer/inhibitor (KP buffer/eserin; BW284c51; GUK-987; GDK-510). In the negative control reaction, we have 120 µL K-P buffer and 100 µL Elman's substrate reagents. Absorbance was measured at **420nm** using a spectrophotometer (BioTek, Cytation 3, Bad Friedrichshall, Germany) for 40 cycles (30 sec interval per cycle, 20min). Using the protein concentration and mass of each sample as well as the difference in absorbance and reaction rate over the selected time frame (linear absorbance selection/time cycle), the enzymatic activity of each sample is calculated using the Microsoft Excel 2007 program.

Technical view 2: To calculate enzyme activity, equation c=n/V is used, where C is concentration of the solution (mol/L), n is moles of substance being dissolved (moles of solute) and V is volume of the solution in liters (L). Mass of protein is expressed in mg unit. Change of absorbance in min is calculated and concentration in minute is assessed (mM) utilizing Lamber Beer Law. Spead of reaction is calculated as change of concentration in minute multiplied by Total volume, and is expressed in nmol/min. Final cholinesterase activity is calculated dividng reaction speed and protein mass, and is expressed in µmol/min/mg protein.

3. Statistical analysis

Data analysis was performed by **IBM SPSS software** version 23.0 for statistical analysis. **Descriptive statistics, Pearson correlation, Linear Stepwise regression and Paired T test** was assessed. Results are expressed as **mean +-SD** and **p value**. Statistical significant correlation was assumed when **p<0.05**.

4. Results

4.1 Participants information

Starting number of participants, **before physical activity,** were **62 individuals**. Difference among participants is based on anthropometry measures, gender, age, blood protein and AChE activity. At the beginning of the study, total **number of female participants** in the study was 47

(75.80%), while total number of male participants in the study was 15 (24,20%). Total **mean age** for male and female was 68.625y (min=59y, max=85y), SD=4.964. Male mean age was 69.93y (min=65, max=80), SD=4.69. Female mean age was 68.22, min=59, max=85, SD=5.021.

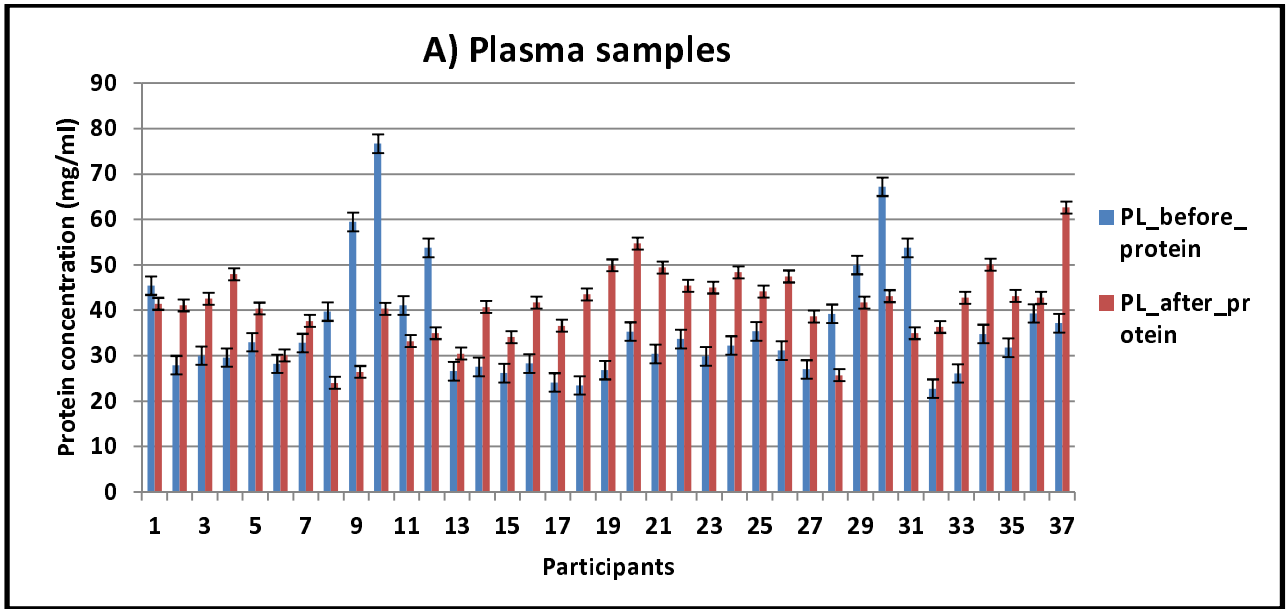
After 3 months of physical activity, final number of participant was 37. Difference among participants is based on gender, age, blood protein and AChE activity. Total number of female participants in the study was 27 (73%), while total number of male participants in the study was 10 (27%). Total mean age for male and female was 68.35y (min=60y, max=80y), SD=68.35. Male mean age was 70.8y (min=65y, max=80y), SD=4.7. Female mean age was 67.44y (min=60y, max=78y), SD=68.3.

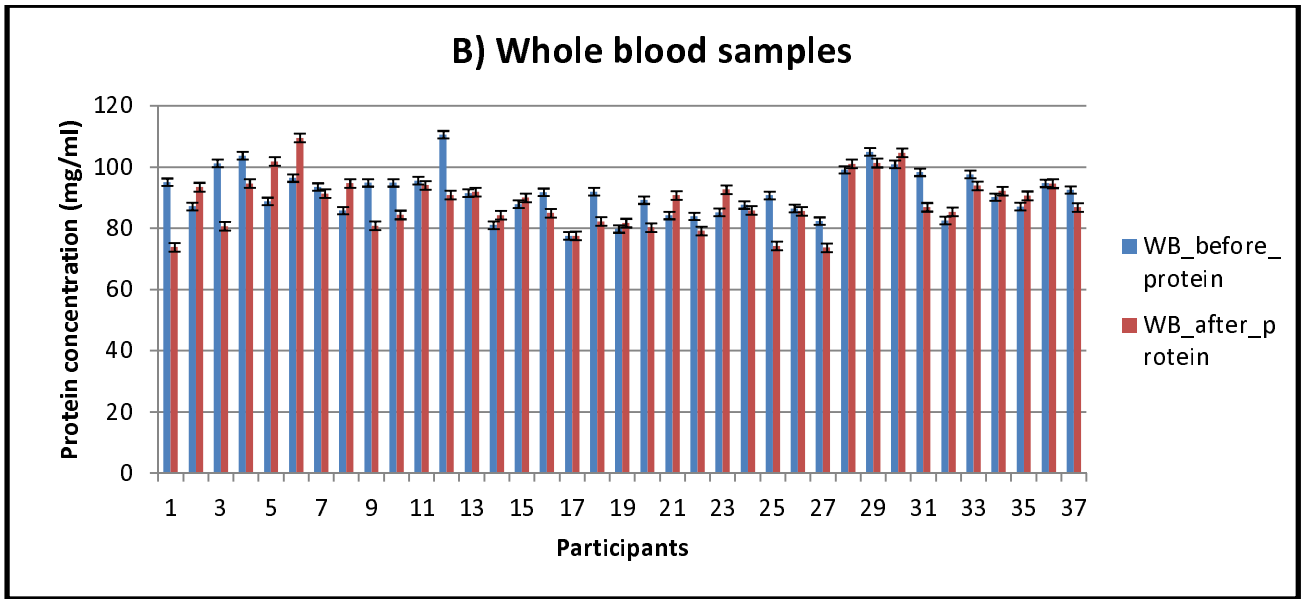
Table 1 shows basic characteristics of the participants based on **Gender** (Male and Female) and **Age** (Group A: 60-70 years and Group B: 70-80 years). Descriptive statistics of the participants include mean and standard deviation value for followed characteristics of **anthropometry, protein /enzyme AChE concentration, inhibitor efficacy before and after 3 months** of physical activity in whole blood and plasma samples. Protein concentration is expressed in mg/ml, while enzyme activity is expressed as nmol/min/mg. All data are parametric, with normal, Gaussian curve distribution. Further statistical analysis employed **parametric** statistical test.

TABLE 1. Participants characteristics	Gender							
	F				M			
	Age				Age			
	Group A (60-70y)		Group B (70-80y)		Group A (60-70y)		Group B (70-80y)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
H (cm)	160.55	6.24	165.88	6.02	174.70	9.91	173.67	2.31
W (kg)	68.36	12.64	67.30	11.24	76.26	10.46	79.80	14.26
BMI	26.58	5.05	24.45	4.00	24.95	2.25	26.39	4.16
WC (cm)	88.02	10.71	84.00	9.41	91.00	8.32	96.25	11.59
HC (cm)	105.31	10.98	104.40	7.41	100.42	4.22	102.25	8.14
FM (kg)	27.17	9.62	23.42	7.80	21.20	5.43	23.67	11.87
MM (kg)	18.63	2.17	19.38	2.59	27.67	3.37	27.62	2.65
PL_before_protein	35.90	12.91	29.24	2.73	39.41	17.40	41.44	5.79
PL_after_protein	40.00	7.73	42.12	6.46	41.75	5.46	43.20	15.11
WB_before_protei n	90.58	7.42	89.43	8.43	92.81	7.25	97.87	5.49
WB_after_protein	86.13	8.20	90.21	10.36	92.30	6.88	95.98	6.81
PL_inducedAChE_ before	.07	.03	.07	.02	.06	.02	.06	.01
PL_eserin 0.1mM_before	.00	.00	.00	.00	.00	.00	.00	.00

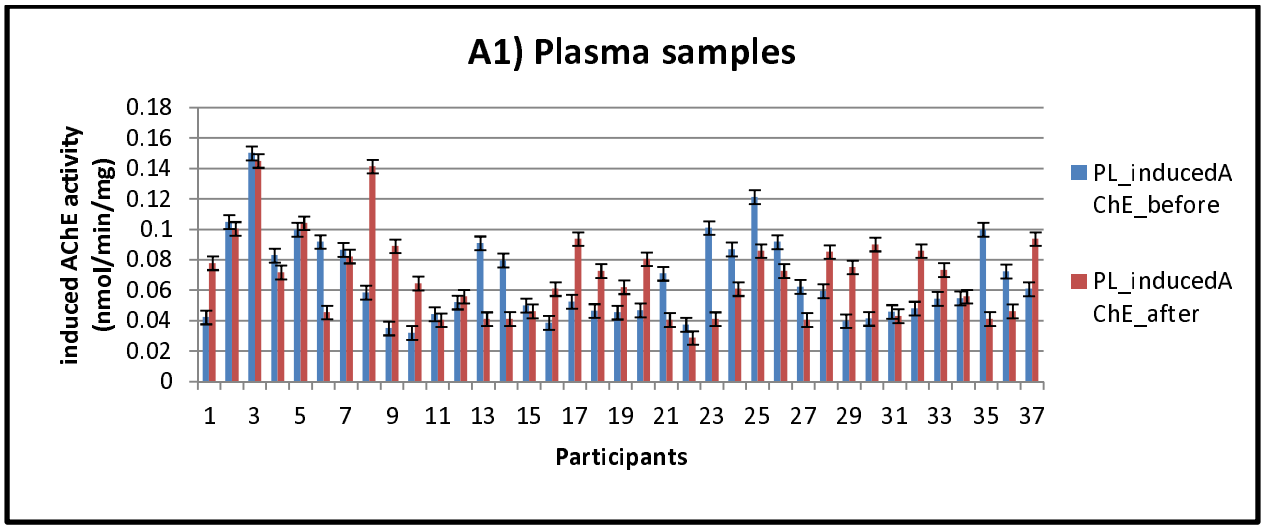
PL_inducedAChE_ after	.07	.03	.06	.03	.07	.02	.08	.02
PL_eserin 0.1mM_after	-.01	.01	-.01	.01	-.01	.01	.00	.00
WB_inducedAChE _before	.08	.03	.07	.02	.06	.03	.06	.02
WB_eserin 0.1mM_before	.00	.00	.00	.00	.00	.00	.00	.00
WB_inducedAChE _after	.21	.24	.17	.02	.19	.04	.17	.04
WB_eserin 0.1mM_after	.01	.00	.01	.00	.02	.00	.03	.03

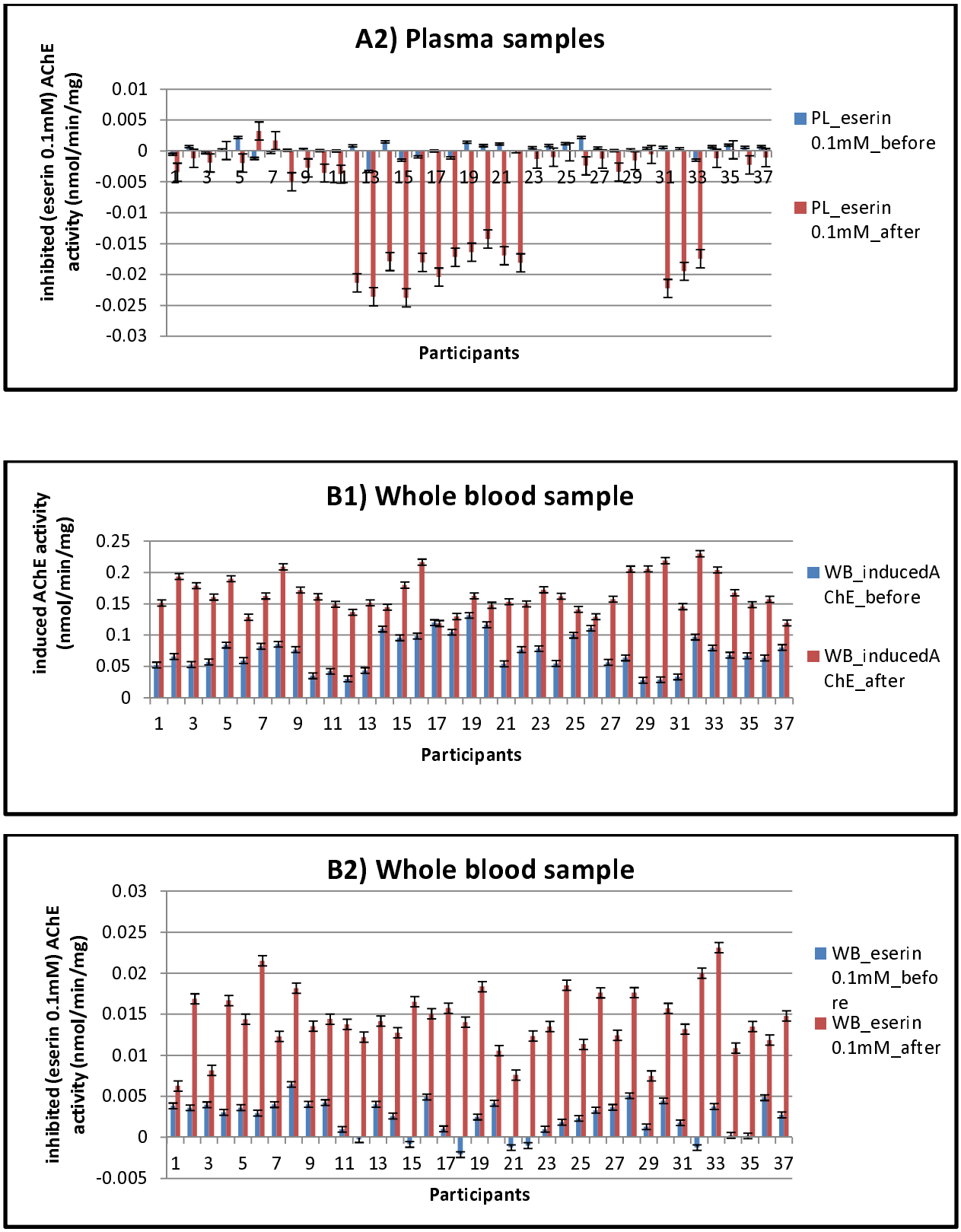
Based on the provided information about mean value from Table 1., it can be concluded that there is a difference between **gender and age group and** for PL and WB samples before and after physical activity for the analyzed parameters (protein concentration and basal enzyme AChE activity). Exception of this connotation is seen for **PL and WB inhibitor (eserin) group before and after physical activity**, indicating the same efficacy of enzyme inhibition. **Graphical representation** of protein concentration and enzyme activity before and after physical activity is presented in the next **graphs 1 and 2**:





Graph1. Protein concentration (mean+-SD) in Plasma and Whole blood samples





Graph 2. Induced and inhibited (eserin 0.1mM) enzyme activity (mean+-SD) in Plasma and Whole blood samples

In order to confirm if this mean difference is statistical significant or not, we employed next parametric **test: Pearson correlation, Linear stepwise regression, T test and One way ANOVA. Pearson correlation**

4.2 Pearson correlation

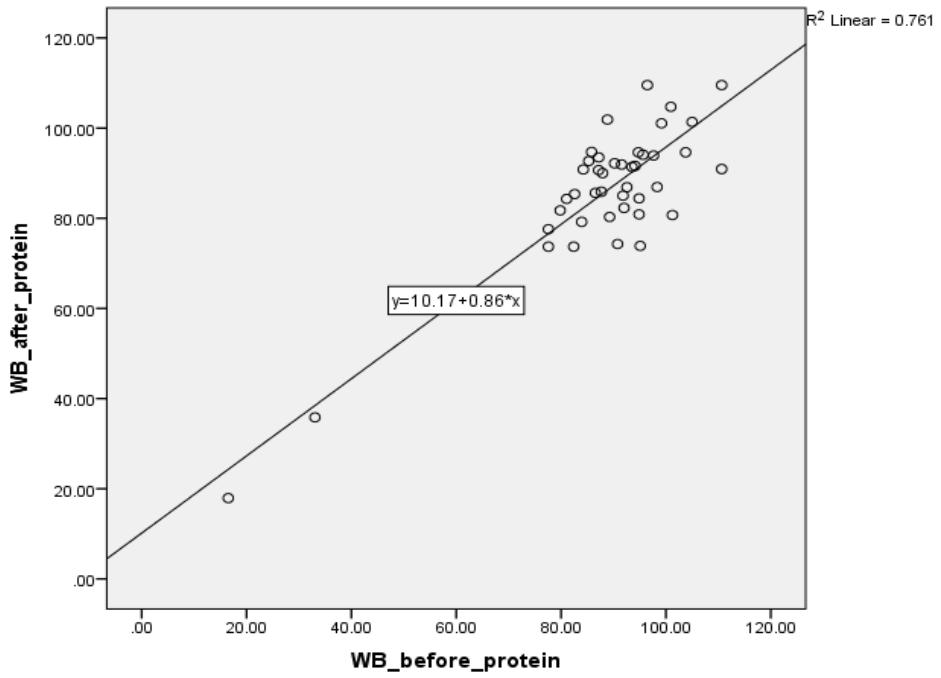
Pearson correlation showed statistical significant linear correlation ($p<0.05$*, $p<0.01$**) for the next variables:

- Age with WB eserin_after*; H*,
- H vs Age*, W**, MM**, PL_basalAChE_before*,
- W vs H**, BMI**, WC**, HC**, FM**, MM**,
- BMI vs W**, WC**, HC**, FM**, PL_basalAChE_before*,
- WC vs W**, BMI**, HC**,FM**, MM**, WB_before_protein*,
- HC vs W**, BMI**, WC**, FM**,
- FM vs W**, BMI**, WC**, HC**,
- MM vs H**, W**, WC**, WB_after_protein*,
- PL_Before protein vs WB_before protein**, PL_basal AChE_before *, WB_basal AChE_before**,
- PL_AFTER_protein vs PL_eserin_before**,
- WB_before_protein vs PL_BEFORE_protein**, WB_AFTER_protein*, WB_basal_BEFORE**, WC*,
- WB_After_protein vs WB_before_protein*, WB_basal_before*, WB_basal_AFTER*, WB_eserin_after*, MM*,
- PL_basal_BEFORE vs PL_before_protein*, PL_eserin_after*, BMI*, H*,
- PL_Eserin_BEFORE vs PL_After_protein*,
- PL_basalACHE_AFTER vs WB_eserin_before**
- PL_Eserin_AFTER vs PL_basalAChE_before*, WB_eserin_before*,
- WB_basal_BEFORE vs PL_before_protein**,WB_before_protein**, WB_after_protein*,
- WB_Eserin_BEFORE vs PL_basal_AFTER**, PL_eserin_after*,
- WB_basalACHE_AFTER vs WB_after_protein**,
- WB_Eserin_AFTER vs Age*, WB_after_protein*,

Results from Pearson correlation indicate **possible linear correlation and strength** between two variables and not a **causation effect**, making it a simple test to calculate and interpret. Interpretation of the results can tell only about association of the investigated variables and not the causal effect of physical activity before and after physical activity.

4.3 Linear stepwise regression

Linear stepwise regression revealed only statistical significant correlation for ***WB_after_protein and WB_before_protein*** which is confirmed with **cross validation** for 20 and 80% sample ($p<0.05$).



Graph3. Linear stepwise regression correlation for WB_protein_before and after physical activity

Unmodified linear regression line for **age and gender** has been estimated, due to the results of independent T test for anthropometric measures. Results of linear stepwise regression in this case presented poorly association between investigated variables due to greater control of the size effect by parameter estimate divided by the standard error.

4.4 T test

Independent T test for anthropometry measures revealed statistical significant correlation ($p<0.05$) for: H, W, WC and MM. These results showed that **age and gender variables** are not significantly correlated, meaning that **age and gender in these examples** have no influence on protein concentration, enzyme AChE activation and inhibition in human blood and plasma samples.

Two tailed, paired sample T test was used to assess the presence of statistical significant correlation between variables for all genders before and after physical activity with 95% confidence interval.

Paired T test, revealed statistical significant correlation ($p<0.01$) between the next pairs before and after physical activity: WB_before_protein vs WB_after_protein, PL_eserin_before vs PL_eserin_after, WB_induced_before vs WB_induced_after, WB_eserin_before vs WB_eserin_after. Paired T test showed that physical activity has effect on protein concentration and enzyme AChE activity depending on the sample type.

4.5 One way ANOVA

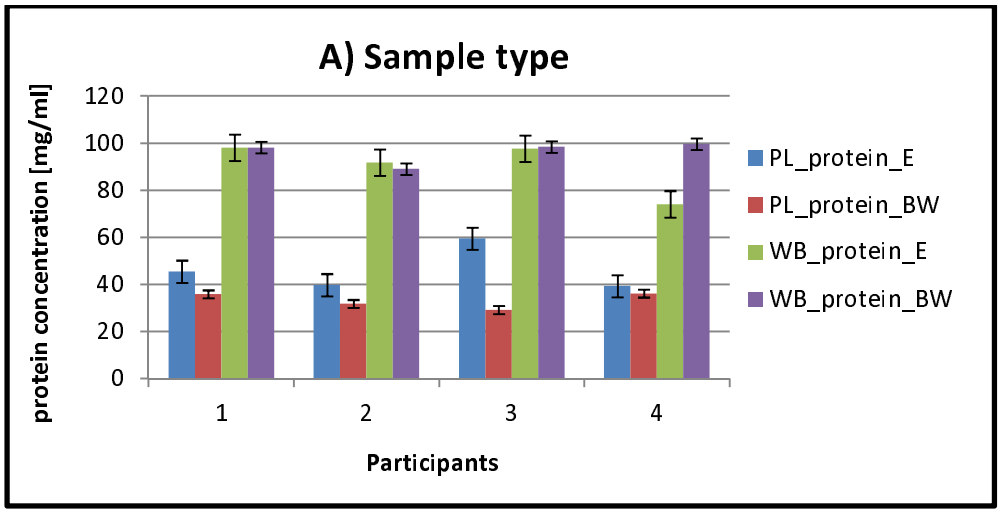
F statistics (one way ANOVA) showed only statistical significant difference between **plasma samples_induced before vs after physical activity**.

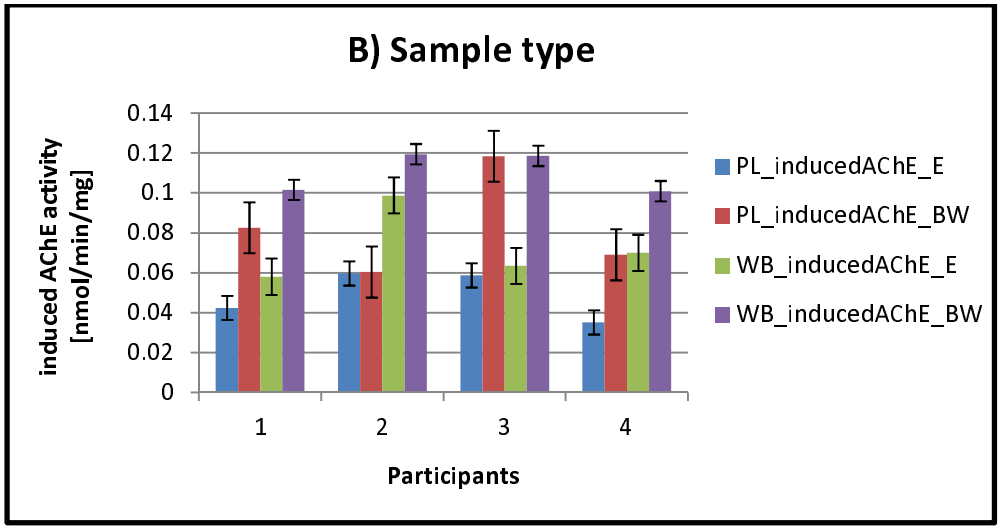
Results of F statistics indicated that physical activity has an effect on AChE activity in plasma samples of participants.

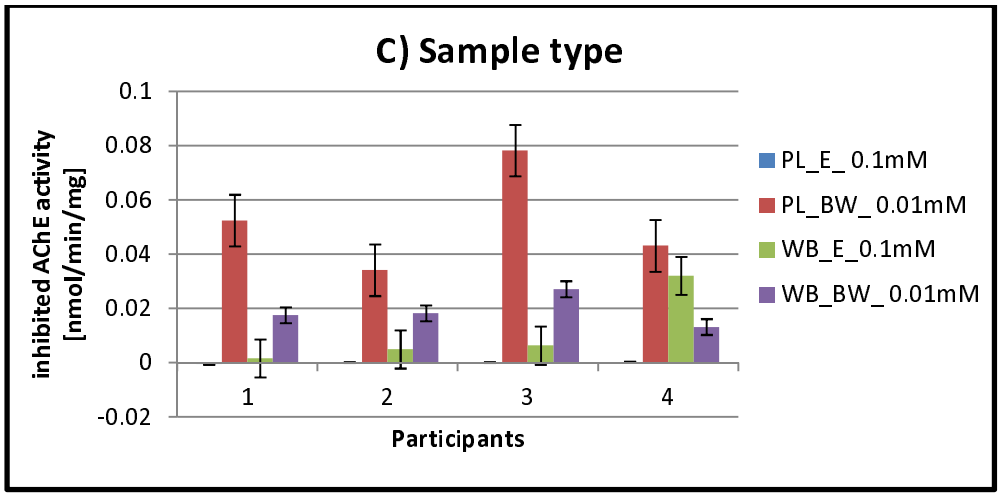
4.6 Inhibitor efficacy (ESERINE and BW284c51)

In order to assess the inhibitor efficacy of two inhibitors (inhibitor BW284c51 (0.01mM) and eserin (0.1mM)) in whole blood and plasma samples, additional experimental study was conducted on total 4 participants, 3 female and 1 male participants.

Difference in eficasy of inhibiton between **eserine and bw284c51, along with protein concentration and basal AChE activity** is present in the **Graph 4**.







Graph4. Measurement of inhibitor efficacy (Eserin, 0.1mM and BW284c51,0.01mM) in whole blood and plasma samples

Based on the results from Graph 4 we can conclude that WB samples contain higher amount of protein concentration, basal AChE activity, in comparing to plasma samples. Inhibitor **Eserin is more potent inhibitor in Plasma samples, while inhibitor BW284c51 is more potent in Whole Blood samples.**

Paired T test showed that there is statistical significant difference ($p<0.05$) in inhibition between eserine 0.1mM and BW284c51 0.01mM inhibitor in Plasma samples. This result is in the accordance of the higher potency of inhibitor eserin in comparing to BW284c51 0.01mM. Based on this notion we used inhibitor eserin for all of our samples.

5. Discussion:

Homeostasis is important factor for maintaining stable state of biochemical and molecular processes of organism. Information about normal physiological processes is of key importance for prevention, follow up, prognosis, and treatment of disease. Understanding source of individual variability is necessary for data interpretation and estimation of expected values during heath and disease. Protein concentration, enzyme activity and inhibitor efficacy in healthy participants can be utilized as a prognostic and diagnostic marker.

Usage of diverse statistical tests has been made to describe association between predictors and potential outcome. Results indicate that it is hard to draw conclusion from just one test, due to different variability of the results and interpretation. Moreover, probability of selecting appropriate statistical test and interpretation of data will depend also on the number of participants and type of data. There is a need for statistic standardization methodology in clinical research.

Although results from our study are not consistent within diverse statistical test, other **study confirm** our main finding, that physical activity influence change of AChE activity, although the study was conducted on younger population and short term activity with significant difference between genders [33]. Moreover, this study is in the accordance to the finding that concentration and activity range is not dependent on age and gender, but it is not in the accordance of usage non parametric tests based on included participants [34].Apart from these studies, available literature lack, indicating a small usefulness of practical information for application in scientific and clinical practice regarding the influence of physical activity on enzyme AChE concentration.

6. Conclusion:

Physical activity represents promising therapeutically strategy for preventing mental disorder, cancer, and cardiovascular diseases. Physical activity can have non-pharmacological effect or adjunctive therapy to diverse disorders.

This is the first study investigating the effect of prolonged physical activity on protein concentration and enzyme AChE activity in whole blood and plasma samples in elderly healthy individuals, utilizing diverse statistical test for results interpretation.

Main conclusion from this study about influence of physical activity on protein concentration and enzyme activity can be derived. Prolonged physical activity has an effect on protein concentration, enzyme activity and inhibition efficacy in plasma and whole blood samples. Interpretations of the results differ among diverse sample type, but the key finding remains. The main disadvantage of our study is number of participants and gender discrepancies. It would be good to increase the number of participants and to compare the results finding on the level of population, include blood and urine parameters and see how prolonged physical activity influence change of homeostatic range. Since all of these biochemical parameters represent the state of organism, they can be used as a potential indicator of disease onset and progression.

There is a need for simple, fast, economic and reliable biomarker after complete understanding changes of values in diverse physiological states. Protein concentration and enzyme AChE activity is one of the potential biomarker for multiple applications. Discovering more potential molecular markers which are specifically activated during physical activity, will be a step closer to appropriate disease treatment and therapy, by tailoring specific training regime for patients with diverse disease states.

However, many challenges remain to be resolved, since physical activity causes complex interactions with diverse organ systems and blood, activations of diverse signal network with protective and harmful mechanism. Future work should emphasize interaction between health and disease including higher number of participants.

List of Abreviations

A

AChE_Acetilcholine esterase, (sinonim: blood, erythrocyte cholinesterase)

AChCl_Acetilcholine chloride

B

BW_BW284c51 inhibitor, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide

BChE_ Butyrylcholinesterase (sinonim: plasmatic, pseudocholinesterase)

C

CRP_C reactive protein

E

E_Eserine, Physostigmine inhibitor

ER_Erythrocites

H

HDL_ High density lipoprotein

L

LDL_Low density lipoprotein

M

mRNA_Mesenger Ribonucleic acid

P

PL_Plasma

S

SNP_Single nucleotide polymorphism

W

WB_Whole blood

References

1. Dvir H, Silman I, Harel M, Rosenberry TL, Sussman JL. Acetylcholinesterase: From 3D Structure to Function. *Chemico-Biological Interaction*. 2010; 187 (1-3):10-22.
2. Soreq H, Seidman S. Acetylcholinesterase-new roles for an old actor. *Nature Review Neuroscience*. 2001; 2(4):294-302. doi:10.1038/35067589.
3. Xi HJ, Wu RP, Liu JJ, Zhang LJ, Li ZS. Role of acetylcholinesterase in lung cancer. *Thoracic Cancer*. 2015; 6(4):390-8. doi: 10.1111/1759-7714.12249.
4. Campanari ML, Garcia-Ayllon MS, Ciura S, Saez-Valero J, Kabashi E. Neuromuscular Junction Impairment in Amyotrophic Lateral Sclerosis: Reassessing the Role of Acetylcholinesterase. Frontiers in Molecular Neuroscience. 2016; 27(9):160. doi: 10.3389/fnmol.2016.00160.
5. World health organisation: Global health observation data. Whorld Health Statistics 2020: Monitoring health for the SDGs. Available at URL: https://www.who.int/gho/publications/world_health_statistics/2020/en/. Accessed: 15.08.2020
6. World health organization. The top 10 causes of death. Available at URL: https://www.who.int/en/news-room/fact-sheets/detail/the-top-10-causes-of-death. Acccessed: 15.08.2020.
7. GBD 2016 Neurology Collaborators. Global, regional, and national burden of neurological disorders, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet Neurology*. 2019; 18(5):459-80. doi: 10.1016/S1474-4422(18)30499-X.
8. Whiteman DC, Wilson LF. The fractions of cancer attributable to modifiable factors: A global review. *Cancer Epidemiology. 2016;* 44:203–21.
9. Rosenbloom DI, Camara PG, Chu T, Rabadan R. Evolutionary scalpels for dissecting tumor ecosystems. *Biochimica et Biophysica Acta*. 2017; 1867(2):69-83doi: 10.1016/j.bbcan.2016.11.005.
10. Brock A, Krause S, Ingber DE. Control of cancer formation by intrinsic genetic noise and microenvironmental cues. *Nature Review Cancer. 2015;* 15: 499–509.
11. Dhama K, Latheef SK, Dadar M, Samad HA, Munjal A, Khandia R, Karthik K, Tiwari R, Yatoo MI, Bhatt P, Chakraborty S, Singh KP, Iqbal HMN, Chaicumpa W, Joshi SK. Biomarkers in Stress Related Diseases/Disorders: Diagnostic, Prognostic, and Therapeutic Values. *Frontiers in Molecular Bioscience*. 2019;6:91. doi: 10.3389/fmolb.2019.00091.
12. Malm C, Jakobsson J, Isaksson A. Physical Activity and Sports-Real Health Benefits: A Review with Insight into the Public Health of Sweden. *Sports (Basel)*. 2019; 23;7(5):127. doi: 10.3390/sports7050127.
13. Heijnen S, Hommel B, Kibele A, Colzato LS. Neuromodulation of Aerobic Exercise-A Review. *Frontiers in Psychology* 2016; 7(6):1890. doi: 10.3389/fpsyg.2015.01890.
14. Stewart LK, Earnest CP, Blair SN, Church TS. Effects of different doses of physical activity on C-reactive protein among women. *Medicine& Science in Sports & Exercise*. 2010; 42(4):701-7. doi: 10.1249/MSS.0b013e3181c03a2b.
15. Benner A, Sadiq NM. Biochemistry, Pseudocholinesterase. [Updated 2020 Aug 16]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK545284/.

16. Rotundo RL. Biogenesis, assembly and trafficking of acetylcholinesterase. *Journal of Neurochemistry*. 2017; 142: 2(2):52-8. doi:10.1111/jnc.13982.
17. Hasin Y, Avidan N, Bercovich D, Korczyn AD, Silman I, Beckmann JS, Sussman JL. Analysis of genetic polymorphisms in acetylcholinesterase as reflected in different populations. *Current Alzheimer Research*. 2005; 2(2):207-8. doi:10.2174/1567205053585909.
18. Rebai M, Kharrat N, Ayadi I, Rebai A. Haplotype structure of five SNPs within the ACE gene in the Tunisian population. *Annals of human biology*. 2006; *33*(3): 319–29. https://doi.org/10.1080/03014460600621977.
19. Duraes F, Pinto M, Sousa E. Old Drugs as New Treatments for Neurodegenerative Diseases. *Pharmaceuticals (Basel)*. 2018; 11(2):44. doi: 10.3390/ph11020044.
20. Murray AP, Faraoni MB, Castro MJ, Alza NP, Cavallaro V. Natural AChE Inhibitors from Plants and their Contribution to Alzheimer's Disease Therapy. *Current Neuropharmacology*. 2013; 11(4):388-413. doi: 10.2174/1570159X11311040004.
21. Krasowski MD, McGehee DS, Moss J. Natural inhibitors of cholinesterases: implications for adverse drug reactions. Canadian Journal of Anesthesiology. 1997;44(5):525-34. doi: 10.1007/BF03011943.
22. Szwajgier D, Borowiec K. Screening for cholinesterase inhibitors in selected fruits and vegetables. *Electronic journal of Polish agricultural universities.* 2012: 15 (2): http://www.ejpau.media.pl
23. Olivera-Bravo S, Ivorra I, Morales A. The acetylcholinesterase inhibitor BW284c51 is a potent blocker of Torpedo nicotinic AchRs incorporated into the Xenopus oocyte membrane. *British Journal of Pharmacology*. 2005; 144(1):88-97. doi: 10.1038/sj.bjp.0705965.
24. Neugroschl J, Sano M. Current treatment and recent clinical research in Alzheimer's disease. *Mount Sinai Journal of Medicine*. 2010; 77(1):3-16. doi: 10.1002/msj.20165.
25. Booth FW, Roberts CK, Laye MJ. Lack of exercise is a major cause of chronic diseases. *Comprehensive Physiology*. 2012; 2(2):1143-211. doi: 10.1002/cphy.c110025.
26. Warburton DE, Nicol CW, Bredin SS. Health benefits of physical activity: the evidence. *Canadian Medical Association Journal*. 2006;174(6):801-9. doi: 10.1503/cmaj.051351.
27. Piech M, Ptaszek B, Teleglow A, marchewka J, Mardyla M. Enzyme activity: acetylcholinesterase and glucose-6-phosphate dehydrogenase in winter swimmers. *Medical Rehabilitation*. 2017; 21 (4). doi: 10.5604/01.3001.0011.6828.
28. Bartha E, Szelenyl J, Szilagyl K, Venter V, Ha T, Haris-Paldi P, Hollan S. Altered lymphocyte acetylcholinesterase activity in patients with senile dementia. *Neuroscience letters*. 1987; 79(1-2):190-94.
29. Farzi MA, Sadigh-Eteghad S, Ebrahimi K, Talebi M. Exercise Improves Recognition Memory and Acetylcholinesterase Activity in the Beta Amyloid-Induced Rat Model of Alzheimer's Disease. *Annals of Neuroscience*. 2019; 25(3):121-5. doi: 10.1159/000488580.
30. Ruiz-Espejo F, Cabezas-Herrera J, Illana J, Campoy FJ, Vidal CJ. Cholinesterase activity and acetylcholinesterase glycosylation are altered in human breast cancer. *Breast Cancer Research and Treatment*. 2002; 72(1):11-22. doi: 10.1023/a:1014904701723.
31. Pedro Martinez-Moreno, Susana Nieto-Ceron, Juan Torres-Lanzas, Francisco Ruiz-Espejo, Isabel Tovar-Zapata, Pedro Martínez-Hernandez, Jose N. Rodríguez-Lopez, Cecilio J. Vidal, Juan Cabezas-Herrera. Cholinesterase activity of human lung tumours varies according to

their histological classification. *Carcinogenesis*. 2006; 27(3):429-36; doi: 10.1093/carcin/bgi250
32. Ferioli M, Zauli G, Martelli AM, Vitale M, McCubrey JA, Ultimo S, Capitani S, Neri LM. Impact of physical exercise in cancer survivors during and after antineoplastic treatments. *Oncotarget*. 2018; 8-9(17):14005-34. doi: 10.18632/oncotarget.24456.
33. Zimmer KR, Lencina CL, Zimmer AR, Thiesen FV. Influence of physical exercise and gender on acetylcholinesterase and butyrylcholinesterase activity in human blood samples. *International Journal of Environmental Health Research*. 2012; 22(3):279-86. doi:10.1080/09603123.2011.634389.
34. Zlatkovic M, Krstic N, Subota V, Boskovic B, Vucinic S. Determination of reference values of acetyl and butyry cholinesterase activities in Serbian healthy population. *Vojnosatelitski pregled*. 2017: 74(8):736-41.

Declaration:

Ethical approval

Experiments were performed according to **ethical standards** and with written consent of the blood donors. The study was given ethical permission from the National Medical Ethics Committee (number 82/07/14). Experimental part of the experiment was done at the Biotechnical Faculty, University of Ljubljana, Slovenia, within the project **J5-7098** and **CEEPUS free mover PhD student program mobility** with the University of Belgrade, Serbia.

Consent for publication

I, Snežana Jovičić (PhD student, Faculty of Biology, University of Belgrade, Serbia), as an only author of this systematic review, give my consent for contained information regarding this manuscript and information about myself to be published in this Journal.

Availability of data material

Data not available.

Competing interest

The autor, PhD student, Snežana Jovičić, declare no competing interest.

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Authors contribution

Author (Snežana Jovičić, PhD student at the Faculty of Biology, University of Belgrade, Serbia), originated idea for this study along with paper and data collection, analysis, interpretation and writing. Snežana Jovičić made all the effort and contribution by herself for the present work and ensures that questions related to the accuracy or integrity of any part of the work are appropriately investigated, resolved, and documented. She is solely responsible for the quality of this produced work.
Author (Snežana Jovičić) approves the final version of the presented manuscript for submission. Snežana Jovičić, **confirms** that this work is original and has not been published elsewhere, nor is it currently under consideration for publication elsewhere.

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