UNIVERSITY OF MONTENEGRO

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A NEW METHOD FOR STRATIFICATION OF THE RISK OF ALZHEIMER'S DISEASE IN PATIENTS IN MONTENEGRO

DOCTORAL DISSERTATION

Podgorica, 2024.

UNIVERZITET CRNE GORE

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NOVA METODA ZA STRATIFIKOVANJE RIZIKA ZA OBOLIJEVANJE OD ALCHAJMEROVE BOLESTI KOD PACIJENATA U CRNOJ GORI

DOKTORSKA DISERTACIJA

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Posveta

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Abstract/Thesis Overview:

Introduction: Pathological and clinical features of Alzheimer's disease (AD) are in temporal discrepancy and currently accepted clinical tests provide the diagnosis decades after the initial pathophysiological events. In order to reach the goal of early detection of this incurable disease, research efforts are directed to the identification of non-invasive, widely accessible biomarkers, which could open the screening possibilities and overcome the main disadvantages of the current AD biomarkers. The present study offers an innovative and broader research context in this regard based on the fact that two different diseases, AD and cancer, have been shown to have inverse incidences. Namely, this recent finding has brought a different and challenging context to the knowledge about shared biological mechanisms involved in these diseases, which are mainly opposite in their nature: AD is characterized by apoptotic cell death and cancer by uncontrolled proliferation. It has been postulated that these mechanisms might actually represent different sides of the same path: if dysregulation of the common AD-CAC signaling pathways leads to a higher risk of cancer, the risk of AD will be reduced in the same individual, or vice versa. However, the precise explanations underlying this negative epidemiological association between these two diseases remain unknown. Small, non-coding RNA molecules – microRNAs (miRNAs), which simultaneously regulate the expression of multiple target genes, are deeply implicated in both diseases and proposed as having the biological complexity to explain such a challenging

relationship between AD and cancer. Being well documented as the leading regulators of cell proliferation and differentiation, migration and apoptosis, which are the main pathological features of cancer and AD, miRNAs may be the key points of the pathophysiological process of both diseases. Additionally, a series of studies have demonstrated their implication in a variety of human brain dysfunctions such as innate immunity, neuroinflammation, dysregulated amyloid metabolism and oxidative stress. In fact, these processes represent the backbone of the different AD theories. Studying the miRNA roles in this disease has thus been recognized as a promising research direction and number of the miRNA interactions with the key genes involved in the pathogenesis of AD has already been identified. The most dominant theories of AD and CAC are based on two closely interconnected processes – innate immunity and inflammation, and thus, they have been chosen as the pathophysiological focus of the present study. Neuroinflammatory signaling pathways and inflammation-related microRNAs (miRNAs) could possibly have a crucial role in AD making them promising potential biomarkers. All the miRNAs selected for this study, not only have a documented role in AD and CAC, but rather, each of them has at least one target involved in immune-related and/or inflammatory pathways: miR-29a, miR-101, miR-125b, miR-146a and miR-155. In addition, the possibility to use these molecules as non-invasive biomarkers contributes to the particular translational value of the whole research context presented.

Objective: Expression of circulatory miRNAs which are deeply involved in the pathogenesis of AD and CAC: miR-29a/b, miR-101, miR-125b, miR-146a, and miR-155 were examined in healthy individuals, those whose cognitive performance on neuropsychological screening was in mild cognitive impairment (MCI) range, patients with AD and CAC. It was hypothesized that these miRNA expression levels could correlate with the level of participants' cognitive decline leading to the identification of molecular signature of early AD. Additionally, it was expected that the expression pattern of selected miRNAs could explain the molecular basis of the inverse incidences between AD and CAC.

Methods: The present study enrolled 54 subjects, out of a total of 75 examined individuals. During the recruitment, of 34 volunteers who felt physically and mentally healthy, 18

individuals were involved in the control group (CTRL-18). Participants who did not report subjective cognitive decline (SCD) but were in the MCI range on Montreal Cognitive Assessment test, formed the low-performance MoCA group (LP-MoCA-9). AD patients were retrospectively recruited at the Neurology Clinic of the Clinical Center of Montenegro (CCM) (AD-12). They were previously diagnosed according to the criteria of the National Institute of Aging and Alzheimer's Association. AD group included patients in the early symptomatic disease stage (EAD) – MCI due to AD and mild dementia stage, (MoCA score $\geq$ 17) and advanced AD (AAD) – moderate and severe dementia (MoCA score < 17). Cognitively unimpaired CAC patients with pathohistologicaly confirmed diagnosis were recruited at the Center for Digestive Surgery – CCM (CAC-15). Standardized questionnaires, physical and neurological examinations, open-ended type of SCD evaluation, neuropsychological screening tests, Geriatric Depression Scale (GDS), as well as biochemical laboratory assessment, were conducted to identify exclusion criteria. Selected miRNAs were extracted from plasma, quantified by qRT-PCR and expression levels normalized by miR-361-5p gene. Statistical data analysis was done with the GraphPad Prism 9 software.

Results: As a control group, this study included volunteers without SCD, but interestingly, neuropsychological screening tests indicated that 33,3% of the apparently healthy subjects were in the MCI range. Neurological and laboratory findings could not explain their neuropsychological performance. However, in the LP-MoCA group, the two circulatory miRNAs, miR-146a and miR-155 were up-regulated compared to the CTRL group ($p < 0.05$). The expression level of miR-146a was also significantly higher in LP-MoCA compared to the AD group ($p < 0.05$). The EAD group also followed this interesting expression pattern for both, miR-146a and miR-155, the same as the heterogeneous AD group. The difference in the expression level of miR-146a and miR-155 between EAD and AAD was not statistically significant. Finally, ROC curve analyses suggested that these miRNAs could serve as non-invasive biomarkers of early cognitive impairment.

Among five examined miRNAs in the present research, only the expression levels of miR-101 in study groups were in agreement with the postulate about the inverse relationship

between AD and CAC. The mean expression values of miR-101 for the CAC and AD groups were on the opposite sides of the range, while the expression level of the CTRL group was in the middle (AD-1.569, CTRL-1.171 and CAC-0.8340). Furthermore, miR-101 expression was significantly higher in AD compared to the CAC group ($p < 0.05$). The CTRL group was not significantly different neither from AD or CAC group. However, the results of ROC curve analysis indicated that on average, a CAC patient will have lower miR-101 expression than 64% of controls, and an AD patient will have upregulated miR-101 expression compared to 63% of people in the general population. This analysis provided the quality score which describes the performance of this AD-CAC inverse correlation model based on miR-101 expression, as a good one. This model was also tested with respect to the disease stage of the AD group. Interestingly, miR-101 expression level was significantly higher in EAD compared to CAC ($p < 0.05$), however, its expression values decreased with the progression of AD towards the later stage, approaching the level of expression in the CAC group. Therefore, no significant difference was observed between AAD and CAC groups, indicating that patients with AAD do not contribute to the significant difference observed between AD and CAC groups.

Conclusions: This study accidentally identified that a certain number of patients with cognitive decline in the Montenegrin population remain undetected. Therefore, neuropsychological screening instruments should be routinely administered to the elderly in Montenegro, even if the patient does not complain of cognitive functioning. The up-regulation of miR-146a and miR-155 could serve as a non-invasive, circulatory biomarkers for the detection of people with CI who are at risk for AD. Together with neuropsychological screening, molecular markers identified by this study, could possibly become routine, easily-accessible tools for screening of the general population for AD.

MiR-101 negatively regulates amyloid precursor protein (*APP*) gene and APP metabolism, therefore, its down-regulation found in AD is understood as a disease-contributing factor. However, the up-regulating trend of miR-101 in early AD and its decline with disease progression found in the present study, suggests that miR-101 might act within the negative feedback mechanism, related to APP metabolism. Given that miR-101 potentially reflects a

progression of amyloid accumulation, it could serve to monitor the effects of amyloid lowering therapy, indicated in the early AD population. Considering miR-101's oncosuppressive role, increased miR-101 expression in long-lasting preclinical and early AD might protect AD patients from cancer. In fact, simultaneous negative regulation of oncogenes and *APP* gene, through the up-regulation of miR-101, as the overlapping point of different signaling pathways, might explain the opposite incidences of AD and CAC.

Keywords: Alzheimer's disease, mild cognitive impairment, neuroinflammation, miR-146a, miR-155, cancer, miR-101, negative feedback mechanism.

Scientific field: Neuroscience – Translational Neuroscience – Alzheimer's disease

PODACI O DOKORSKOJ DISERTACIJI

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Uvod: Pojava patoloških obilježja Alchajmerove bolesti (AB) i njeno kliničko ispoljavanje, su u vremenskoj diskrepanci. Stoga, kliničkim testovima koji se aktuelno koriste, dijagnoza se postavlja decenijama nakon inicijalnih patofizioloških događaja. Kako bi se dostigao cilj ranog otkrivanja ove neizlječive bolesti, istraživački napori su usmjereni ka identifikovanju neinvazivnih, široko dostupnih biomarkera, koji bi otvorili mogućnosti za skrining i tako prevazišli nedostatke aktuelnih biomarkera. Sa tim ciljem, ova studija nudi inovativan i širok istraživački kontekst, koji se zasniva na činjenici da obolijevanje od dviju različitih bolesti, AB i karcinoma, karakteriše inverzna incidenca. Naime, ovo nedavno otkriće je donijelo jedan drugačiji, izazovan kontekst u odnosu na prethodna saznanja o zajedničkim biološkim mehanizmima ovih bolesti, koji su takođe, većinom, suprotne prirode: AB se karakteriše ćelijskom smrću apoptozom, a kancer nekontrolisanom ćelijskom proliferacijom. Stoga, smatra se da bi ovi mehanizmi zapravo, mogli predstavljati suprotne strane iste patofiziološke kaskade, objašnjavajući tako i suprotne incidence AB i CAC: ukoliko disregulacija zajedničkih signalnih puteva ovih dviju bolesti vodi povećanom riziku od kancera, rizik od AB će kod iste osobe biti redukovan, odnosno suprotno. Međutim, precizna objašnjenja koja

se nalaze u osnovi inverzne epidemiološke povezanosti ovih dviju bolesti, još nijesu poznata. Male, nekodirajuće RNK molekule – mikroRNK (miRNK), koje ostvaruju negativnu regulaciju ekspresije gena, simultano na višestruke targete, duboko su uključene u patogenezu obiju bolesti i smatra se da posjeduju biološku kompleksnost koja bi mogla ponuditi objašnjenje ovako izazovnog odnosa AB i kancera. S obzirom na dobro dokumentovane uloge miRNK, kao vodećih regulatora procesa ćelijske prolieracije i diferencijacije, migracije i apoptoze, koji su među glavnim karakteristikama AB i kancera, smatra se da bi ove molekule mogle objasniti ključne aspekte patofizioloških procesa obiju bolesti. Uz to, serije istraživanja su pokazale njihov značaj u mnogim aspektima moždanih disfunkcija, a koji su u osnovi različitih teorija AB: urođeni imunitet, neuroinflamacija, deregulacija metabolizma amiloida i oksidativni stres. Stoga je proučavanje uloga miRNK u ovoj bolesti, prepoznato kao obećavajući pravac istraživanja. Štaviše, jedan broj miRNK molekula su već i prepoznate kao potencijalni biomarkeri ove bolesti. Dominantne teorije AB i karcinoma se baziraju na dva međusobno blisko povezana procesa – urođenom imunitetu i inflamaciji, a neuroinflamatorni signalni putevi i sa inflamacijom povezane miRNK, mogle bi imati krucijalnu ulogu u AB, čineći ih potencijalnim biomarkerima. Stoga su kao patofiziološki fokus ove studije odabrane one miRNK, koje ne samo da imaju dokumentovane uloge u AB i CAC, već svaka od njih ima najmanje jedan ciljni gen koji je uključen u puteve povezane sa imunološkim i/ili inflamatornim odgovorom: miR-29a, miR-101, miR-125b, miR-146a and miR-155. Uz to, mogućnost da se ove molekule koriste kao neinvazivni biomarkeri, posebno doprinosi translacionoj vrijednosti prezentovanog istraživačkog konteksta.

Cilj: Ekspresija cirkulišućih miRNK, kompleksno uključenih u patogenezu AB i kolorektalnog karcinoma (CAC): miR-29a/b, miR-101, miR-125, miR-146a and miR-155, ispitivana je kod zdravih pojedinaca, onih kod kojih je Montrealskom skalom za procjenu kognicije (eng. Montreal cognitive Assessment - MoCA) detektovan skor u opsegu za blagi kognitivni poremećaj (eng. mild cognitive impairment – MCI), kao i kod pacijenata sa dijagnostikovanim AB i CAC. Postavljena je hipoteza da će ekspresija navedenih miRNK biti u korelaciji sa stepenom kognitivnog deficita ispitanika, što bi moglo biti od značaja u

identifikovanju molekularnog potpisa rane AB. Takođe, očekuje se da bi obrazac ekspresije selektovanih miRNK kod pacijenata sa AB i CAC, mogao objasniti molekularnu osnovu inverzne incidence ovih dviju bolesti.

Metodologija: U ovu studiju su uključena 54 ispitanika, od ukupno 75 regrutovanih pojedinaca. Od 34 volontera koji su se osjećali fizički i mentalno zdravim, njih 18 je bilo uključeno u kontrolnu grupu (CTRL-18). Kod 9 učesnika koji subjektivno nijesu imali kognitivni deficit, kognitivne performanse na MoCA testu su bile u rasponu za MCI (eng. low-performance MoCA (LP-MoCA)-9). Pacijenti kod kojih je dijagnostikovana AB regrutovani su retrospektivno, u Klinici za neurologiju Kliničkog centra Crne Gore (KCCG) (AD-12). Ovim ispitanicima je dijagnoza prethodno postavljena u skladu sa kriterijumima Nacionalnog instituta starenja i Alchajmerove asocijacije. AD grupa je uključivala pacijente u ranom simptomatskom stadijumu bolesti (early Alzheimer's disease – EAD) – MCI uzrokovan AB i stadijum blage demencije (MoCA skor $\geq$ 17) i uznapredovaloj AB (advanced Alzheimer's disease – AAD) – umjerena i teška demencija (MoCA score < 17). Pacijenti sa CAC i bez kognitivnog oštećenja sa patohistološki potvrđenom dijagnozom, bili su regrutovani u Centru za digestivnu hirurgiju KCCG (CAC-15). Standardizovani upitnik, fizikalni i neurološki pregledi, neuropsihološki skrining testovi, gerijatrijska skala depresije, kao i biohemijska laboratorijska procjena, sprovedeni su sa ciljem identifikovanja isključujućih kriterijuma. Odabrane miRNK su bile izolovane iz plazme, kvantifikovane metodom lančane rekacije polimeraze u relnom vremenu i nivoi ekspresije normalizovani upotrebom miR-361-5p gena.

Rezultati: Ovom studijom su u kontrolnu grupu uključeni volonteri bez subjektivnog kognitivnog deficita, ali interesantno, na osnovu skora na MoCA testu, 33,3% njih, naizgled zdravih ispitanika, pripadalo je kategoriji MCI. Neurološki pregled i laboratorijske pretrage nijesu mogli objasniti učinak ovih pojedinaca na neuropsihološkim testovima. Međutim, u LP-MoCA grupi, nivo ekspresije dviju cirkulišućih miRNK, miR-146a i miR-155, bio je značajno viši u odnosu na kontrolnu ($p < 0.05$). Ekspresija miR-146a je bila u značajnom porastu u LP-MoCA i u odnosu na AD grupu ($p < 0.05$). U odnosu na EAD grupu, rezultati su pokazali isti obrazac ekspresije za obje miRNK - miR-146a i miR-155, kao i u odnosu na

čitavu, heterogenu AD gupu. Razlika u nivou ekspresije ovih miRNK između EAD i AAD grupa nije bila značajna. Konačno, analiza krivulje operativnih karakteristika (eng. Receiver operative characteristic curve – ROC curve), upućuje da bi miR-146a i -155 mogle služiti kao neinvazivni biomarkeri ranog kognitivnog oštećenja.

Među pet ispitivanih miRNK, samo je nivo ekspresije miR-101 u studijskim grupama bio u saglasnosti sa postulatom o inverznom odnosu između AB i CAC. Srednje vrijednosti ekspresije miR-101 za CAC i AD grupe bile su na suprotnim stranama opsega, dok je nivo ekspresije CTRL grupe bio u sredini (AD – 1.569, CTRL – 1.171 and CAC – 0.8340). Štaviše, ekspresija miR-101 bila je značajno veća u AD, u poređenju sa CAC grupom ($p < 0.05$). Nivo ekspresije ove miRNK u CTRL grupi se nije razlikovao u odnosu na AD i CAC grupe. Međutim, rezultati analize ROC krive upućuju na to da će u prosjeku CAC pacijent imati nižu vrijednost ekspresije miR-101 nego 64% zdravih pojedinaca, a AD pacijent bi imao povišenu regulaciju miR-101, u poređenju sa 63% opšte populacije. Ovom analizom je obezbijeđen kvalitativni skor, kojim je performansa ovog AD-CAC modela baziranog na ekspresiji miR-101, procijenjena kao dobra. Model je takođe testiran i u odnosu na stadijum bolesti AD grupe. Interesantno, ekspresija miR-101 bila je značajno veća u EAD u poređenju sa CAC ($p < 0.05$). Vrijednosti su se smanjivale sa progresijom AB, približavajući se nivou ekspresije CAC grupe, stoga nije opservirana značajna razlika između AAD i CAC grupa.

Zaključci: Ovim istraživanjem je otkriveno da u crnogorskoj populaciji nije prepoznat određeni broj pojedinaca sa kognitivnim deficitom. Stoga bi neuropsihološke skrining testove trebalo rutinski primjenjivati kod pojedinaca starije životne dobi u Crnoj Gori, nezavisno od žalbi na kognitivne funkcije. Povećana ekspresija miR-146a and miR-155, mogla bi predstavljati neinvazivni cirkulišući biomarker za detekciju pojedinaca sa kognitivnim deficitom, koji su u riziku za AB. Zajedno sa neuropsihološkim skriningom, ovi molekularni markeri bi mogli postati rutinska, neinvazivna sredstva za rano otkrivanje AB u opštoj populaciji.

MiR-101 negativno reguliše ekspresiju amiloid prekursor protein (*APP*) gena i APP metabolizam, stoga je njena smanjena regulacija koja karakteriše AB, shvaćena kao faktor koji doprinosi nastanku bolesti. Međutim, trend povećanja nivoa ekspresije miR-101 u EAD,

kao i njeno opadanje sa progresijom bolesti otkriveno u ovoj studiji, sugeriše da bi miR-101 mogla djelovati u okviru mehanizma negativne povratne sprege, koji je u vezi sa APP metabolizmom. Uzimajući u obzir da miR-101 potencijalno reflektuje progresiju akumulacije amiloida, mogla bi služiti i u monitoringu efekata amiloid-redukujuće terapije, indikovane u EAD populaciji. Uzimajući u obzir onkosupresivnu ulogu miR-101, njena potencijalno povećana ekspresija u dugoj pretkliničkoj i ranoj fazi AB, mogla bi zaštititi AB pacijente od kancera. Zapravo, simultana negativna regulacija onkogena i *APP* gena, kroz povećanu regulaciju miR-101, kao tačke preklapanja različitih signalnih puteva, mogla bi objasniti inverzne incidence AB i CAC.

Ključne riječi: Alchajmerova bolest, blagi kognitivni deficit, neuroinflamacija, miR-146a, miR-155, kancer, miR-101, APP metabolizam, mehanizam negativne povratne sprege.

Naučna oblast: Neuronauke – Translacione neuronauke – Alchajmerova bolest

List of abbreviations:

- 3'UTR – 3' untranslated region
- AAD – advanced AD
- Ach – acetylcholine
- AD – Alzheimer's disease
- AD-C – clinical manifestations of Alzheimer's disease
- AD-P – pathological characteristics of Alzheimer's disease
- AH – amyloid hypothesis
- APOE – ε4-apolipoprotein E ε4 allele
- AβPP – amyloid-β protein precursor
- ARIA – amyloid-related imaging abnormalities
- ATN – amyloid, tau, neurodegeneration
- Aβ 40/42 – amyloid-β protein consisting of 40-42 amino acids
- Aß – amyloid-β
- BACE – beta-site APP-cleaving enzyme
- BDNF – brain derived neurotrophic factor
- BMI – body mass index
- CAC – colorectal adenocarcinoma
- CCM – Clinical Center of Montenegro
- CD4 – T-cell surface glycoprotein CD4
- CI – cognitive impairment
- CNS – central nervous system
- COVID-19 – Coronavirus disease 2019
- COX-2 – cyclooxygenase-2
- CR – cognitive reserve
- CSF – cerebrospinal fluid
- CSF1R – macrophage colony-stimulating factor 1 receptor;
- CTRL – control group
- CU – cognitively unimpaired

- EAD – early AD
- ECA109 – human esophageal squamous cancer cell line
- EoAD – early onset Alzheimer's disease
- FADD – FAS-associated via death domain gene
- FDA – Food and Drug Administration
- FDG – fluorodeoxyglucose
- GDS – 15-geriatric depression scale-15
- GFAP – glial fibrillary acidic protein
- GWAS – genome wide association studies
- HeLa – Henrietta Lacks (epithelial cell line)
- IFN – γ-interferon-gamma
- IL-1 – interleukin 1
- IL-18 – interleukin-18
- IL-6 – interleukin-6
- IL1B – interleukin 1B
- KEGG – Kyoto Encyclopedia of Genes and Genomes
- LATE – age-related TDP-43 encephalopathy
- LoAD – late-onset Alzheimer's disease
- LP-MoCA – low-performance Montreal Cognitive Assessment
- MCI – mild cognitive impairment
- mRNA – messenger ribonucleic acid
- MiRNA – small, non-coding ribonucleic acid molecules-microRNAs
- MMSE – Mini-Mental State Examination
- MoCA – Montreal Cognitive Assessment
- MRI – magnetic resonance imaging
- mRNA – messenger RNA
- NFKB1 – Nuclear factor NF-kappa-B p105 subunit
- NFL – neurofilament light
- NIA-AA – National Institute of Aging-Alzheimer's Association
- NMDA – N-methyl-D-aspartate

- P-tau – phosphorylated aggregated tubulin associated unit (tau)
- PALD1 – phosphatase domain containing, paladin 1
- PET – positron emission tomography
- PICALM – phosphatidylinositol-binding clathrin assembly protein
- PIN1 – peptidylprolyl cis/trans isomerase, NIMA-interacting 1 (gene and protein)
- PSEN1 – presenilin 1
- PSEN2 – presenilin 2
- PTGS2 – prostaglandin-endoperoxide synthase 2 gene
- qRT-PCR – quantitative real-time polymerase chain reaction
- ROC curve – receiver operating characteristic curve
- ROS – reactive oxygen species
- SCD – subjective cognitive decline
- SCDI – Subjective Cognitive Decline Initiative
- SNP – single nucleotide polymorphisms
- STRING – Search Tool for the Retrieval of Interacting Genes
- T-tau – total tau
- Tau – aggregated tubulin associated unit
- TDP-43 – Transactive response DNA binding protein-43
- TNF – tumor necrosis factor
- TNF – α-tumor necrosis factor
- TP53 – tumor protein p53
- TREM2 – triggering receptor expressed on myeloid cells 2
- YWHAZ – 14-3-3 protein zeta/delta

PREFACE TO THE DOCTORAL DISSERTATION

The phenomenon of aging has always been a challenge for man. His strong effort to fully understand and delay it fosters him to research and succeed in curing the diseases of aging. However, there are still challenges that have not been overcome. One of them is Alzheimer's disease (AD), which impoverishes the personality and takes away the identity and integrity acquired during life making the biological existence of a man terrible until the fatal outcome.

Global increasing incidence of AD, as well as its seriously negative consequences on the individual, familial, institutional and socio-economic level, have positioned this disease among the leading biomedical priorities, in the past few decades. Doubled lifespan expectancy in the world, since the beginning of the XX century, is the dominant cause of the growing trend of disease of aging. Together with such demographic trend, an absence of a causal therapeutic solution for AD and fatal outcome, have strongly fostered the research in this field.

A significant breakthrough in understanding pathophysiological concept, development of biomarkers and disease-modifying therapy is evident. However, a unified explanation, pathophysiological and molecular signature of AD, which would open the possibility for the development of causative therapy, remains a challenge. In addition, the application of the knowledge achieved is still not successful. Despite the revolutionary advance of detecting AD neuropathological changes before clinical manifestations, by the biomarkers, it is clear that they will not be included in the diagnostic criteria mainly due to invasiveness or unavailability of methods used for their detection. Additionally, the new, disease-modifying therapy, intended for patients in the early stages of the disease, requires the availability of sophisticated imaging for the identification of eligible patients. Therefore, the questions arise: "How can we expect early diagnosis in asymptomatic AD? Who are the new therapeutic modalities actually intended for?" Currently, they are intended for the lucky minority patients who have the opportunity to enter the diagnostic procedure in specialized, large memory clinics. Thus, the identification of new, widely available, simple and non-invasive biomarkers which would reflect the pathophysiological process of AD is urgent.

Furthermore, literature evidence implies that the significance of cognitive problems in the wide population has not been sufficiently recognized and that the stigmatization of the AD population is still widespread. These aspects cause problems regarding AD detection, even in the symptomatic stage. Thus, it needs to be emphasized, that continuous education, raising awareness of the importance of early disease recognition and the fight against stigma must not be overshadowed by large-scale research tasks of discovering biomarkers and new treatments.

The global negative context regarding AD seems to be emphasized in the Montenegrin population. Concerning characteristics of demographic development, indicate that the trend of demographic aging has been present in Montenegro for about half a century. Moreover, it is estimated that our country will continue to be exposed to such an impact in the first half of the 21st century. Additionally, health statistics in the AD field have not been still adequately implemented. According to the available, limited data of the Institute of Public Health, there is a serious concern that currently AD in Montenegro is underdiagnosed and that the number of people with this disease, requiring care, might be much higher. These facts indicate that it would be necessary to place AD as one of the health and research priorities in Montenegro.

In the inevitability of a fatal outcome - once AD begins, the fight against this disease, for now, is actually the fight for its early recognition. It is the battle for the dignity of the human being living with AD. It is the battle to prolong the quality of life and delay the negative outcome. This scientific research is precisely an attempt to identify biological indicators of the very beginning of the disease, which will be easily and widely usable, enabling an early diagnosis of AD. At the same time, they will be potentially the basis for a better understanding of the pathological context of this disease, and hopefully, the path to its causal treatment.

CONTENTS

1. INTRODUCTION:

1.1. Current status of knowledge regarding Alzheimer's disease

Life expectancy has doubled in the world since the beginning of the 20th century leading to an increased incidence of Alzheimer's disease (AD), as a disease of the elderly (1). Currently, 50 million people in the world have been affected by this progressive and ultimately fatal neurodegenerative disorder. Considering the fact that age is the main risk factor for AD, it is expected that this number will reach 139 million by the year 2050 (2). Namely, it is estimated that there will be twice as many people over the age of 65 as children under the age of 5 (3). Precisely, 10% of those who are 65 years and older are thought to have AD (1). However, it should be noted that the incidence of AD decreases in people who are 90 years of age or older (4). At that age, hippocampal sclerosis is even more common but develops as age-related transactive response DNA binding protein-43 (TDP-43) encephalopathy (LATE), resulting in a clinical manifestation often misattributed to AD (5,6). Interestingly, a meta-analysis that explored the incidence of clinical AD in Europe found that it decreases as the age strata explored increase (7). Literature evidence indicates that besides age, gender is one of the most important determinants of the current AD epidemiological status (8,9). It has been reported that an individual's reproductive history and changes in levels of female hormones throughout life contribute to the higher risk for AD in women (10). Therefore, more than 65% of people diagnosed with AD of late-onset are females (8). Moreover, the impact of some of the known AD risk factors, like having apolipoprotein E ε4 allele (+*APOE* - ε4), seems to be stronger in women than in men (11). Eventually, longer life expectancy of women should not be ignored as a potential cause. AD is responsible for 60-70% of dementias, which is the fifth cause of death globally (12,13). The latest figures of this kind show that the mortality rate from AD in Europe in 2013, was 45.2 per 100,000, which is doubled compared to 1994 (14). The official data for the United States indicate that AD was the sixth-leading cause of death in 2019 and the seventh-leading cause of death during the Coronavirus disease 2019 (COVID-19) pandemic in 2020 and 2021 (1). Not only the mortality rate is generally high but reported deaths from AD in the American population increased by more than 145% between 2000 and 2019, in contrast to the deaths from stroke,

heart disease, and HIV – which have decreased (1). This certainly reflects an increasing problem of AD morbidity and mortality; however, it should be considered that deaths may be also more documented in the era of increased AD awareness among healthcare professionals. This global, increasing trend of AD is independent of socio-economic status, and the financial burden due to AD is in the range of cardiovascular diseases and cancer (15). Not only the expenses for AD patients are considered prohibitively high (16), but also, due to the nature of AD, the workload of family members and caregivers becomes a problem. Negative outcomes regarding the mental and physical health of the burned-out family members (1,17,18), as well as expenses and the number of hours devoted to AD patients (1,15), are clearly on the rise.

Despite the growing trends of morbidity, mortality, and burden on health systems due to AD, the most recent survey conducted in the American population indicated a low level of awareness of mild cognitive impairment (MCI) (1) - a condition that should be a warning sign for the potential development of AD, and should represent a clear indication to consult a medical expert. This implies that one important aspect of the whole problem may be neglected. Namely, progress in medical research and services of the health systems is important, but the education of the general population is equally important. Late recognition of the disease and delayed medical consultation are likely to be the root cause of the problem that AD represents globally (19). In support of this, dementia incidence in some counties was found to be in decline in specific age groups, due to the implementation of long–term societal strategies, regarding access to healthcare and education (20,21).

In Montenegro, health statistics in the AD field have not been still adequately implemented. Monitoring and studying the status of this part of the Montenegrin population, as well as the quality of work of health services and health policy planning, are primarily limited by the fact that there is no official collection of data, that is, the Health Register of AD (22). However, Statistical Yearbooks as the regular annual publications of the Institute of Public Health, provide some insight into data on registered diseases in health institutions. The analysis of the available figures for AD indicates that around 0,05% of the Montenegrin population was diagnosed with AD each year, from 2016 to 2019 and 0,06% in 2020 (23).

Interestingly, the deeper analysis of the morbidity in the hospitals and outpatient health services, which are available for the year 2020, shows that all the registered AD diagnoses for that year are coming from primary health care services (24). This fact calls into question the process of work related to cognitive pathology at Primary health care institutions, that is, referring these patients to the expert level for diagnosis. Furthermore, the fact that there are 0.4% of patients registered with dementia as a separate category is confusing considering that AD is responsible for 60-70% of dementia diagnoses (23). Interestingly, available data on the use of medicines in Montenegro do not indicate the consumption of drugs intended for symptomatic treatment of AD, which are currently available (25). Altogether, these data introduce dilemma into consideration and estimation of the number and accuracy of diagnosed AD cases and, finally, strongly imply the need for the thorough and careful systematization of data, that is, the foundation of the Health Register for AD. The analysis of data on mortality from AD, from 2010 to 2019, clearly points to a growing trend, especially since 2014 (23). Although we do not have this volume of data on AD morbidity for the same period, it is noted that the trend of the registered diagnoses was mostly stable and that the mortality trend is increasing (23). That could be explained by the fact, that in the last 6-7 years, AD awareness has increased so that AD as a cause of death was more documented. In addition, the clinical application of AD diagnostic criteria, revised in 2011 (for the first time since 1989), came to life in the following years, so probably, the disease was better recognized and diagnosed. Nevertheless, all the indicated percentages are much lower compared to other European countries. Therefore, there is a serious concern that currently, AD in Montenegro is underdiagnosed and that the number of people with this disease, requiring care, might be much higher.

On the other hand, national guidelines for good clinical practice, for healthcare professionals and institutions, do not address the topics of prevention and management of cognitive problems and dementia (26). Furthermore, there are no national strategies to highlight the importance of early recognition and timely treatment, while many European countries have a progressive health policy in this regard and defined strategies of action (27). In addition, the programs and activities of the Institute of Public Health are very modest in this regard

(28). However, over the last decade, the activities of the non-governmental sector have started to increase, which has raised the quality of care for patients with dementia through numerous projects and associations with state institutions and health professionals. They initiated the opening of a number of counseling centers, and finally, positioned Montenegro on the European map of official associations, dealing with patients suffering from AD. So, nevertheless, it can be concluded that Montenegrin society is gradually progressing and maturing in this sense.

1.2. Pathophysiological context of Alzheimer's disease

The decryption of the complex neurodegenerative process of AD, and particularly its link with the emergence of the clinical syndrome, probably represents the most challenging and the most important aspect of the research in the AD field. It is the key that will enable the development of biomarkers for the disease, as well as successful therapeutic modalities. Although progress in understanding the pathophysiology is evident, a unified explanation has not yet been reached. Several theories bring many useful but often independent arguments, which are not successfully interconnected. So, not only do we need to identify initial and substantial events of AD pathogenesis, but maybe, the many answers are already present among all the valuable pieces of evidence, that need to be put together. Unlike the key causal processes, pathomorphological features of AD, as well as the dynamics of their occurrence, have been unambiguously demonstrated (29,30).

The well-known neuropathological substrate of AD, described for the first time at autopsy studies more than 50 years ago, is represented by extracellular accumulation of amyloid–β (Aβ) protein and intracellular deposition of aggregated tubulin associated unit (tau) protein and phosphorylated tau (p-tau) (29,31,32). It is considered that pathological imbalance of amyloid production and clearance results in its extracellular aggregation in the form of plaques, as well as in its toxicity, which induces further pathological changes (33,34). Pathological phosphorylation and aggregation of tau protein lead to the formation of neurofibrillary tangles and neuropil threads into the neurons (29,31,32). More importantly,

research has also revealed dynamic aspects of tau pathology, closely related to immune response and neuroinflammation. Namely, extracellular tau accumulation has been demonstrated, as well as its active spread from neuron to neuron (35,36). Therefore, one of the current research ideas in the AD field is targeting extracellular tau, in order to prevent its subsequent intracellular accumulation (37).

Mutations in genes that regulate the production of amyloid proteins - amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*), are first identified in AD patients with familial, early onset disease form (38,39). Development of the sporadic AD has been influenced by genetic, but also by environmental factors (39,40,41). Among more than 20 genetic contributors to the increased risk of the late disease form, +*APOE* - ε4 is considered the strongest risk factor for the development of sporadic AD (39,42). Impairment of amyloid and tau cascades have been related to the effects of this genotype (43). Although its presence may account for about half of the patients with sporadic AD, it is assumed that ultimately, environment is the critical factor that converts the risk into a disease (as it is discussed in more details in the section 1.4.).

Temporal ordering of the pathological events in AD has been proposed by several research groups, but many explanations are incomplete. However, genetic discoveries in familial forms of early-onset AD (EoAD) indicate that the initiating molecular events begin with disordered Aβ metabolism (39,44). It has been unequivocally confirmed that amyloid plaques occur decades before the disease is clinically manifested (44). Research data on tau pathology also speak for its presence a decade or more before the clinically evident disease (45), but not as early as amyloid accumulation. Recent works highlight that the later events, like the regional progression of atrophy, correlate with tau, but not amyloid accumulation (46). While amyloid theory has been based on EoAD, there are assumptions that tau pathology may be the cause of the late-onset AD (LoAD) (47). Nevertheless, current research supports the interconnection of amyloid and tau pathophysiological cascades in AD (48). Eventually, some of the crucial AD pathophysiological processes identified in genome-wide association studies (GWAS) are dominantly involved in innate immunity and neuroinflammation, cellular energetics, and proteolysis, but not in amyloid or tau pathways, at least not directly

(49,50). There is no unified explanation of the precise role of each proposed mechanism or order of events, and it is only clear that their synergistic action leads to a neuronal injury and dystrophic neuronal process until its loss (51,52). Atrophy is, thus, the dominant structural pathological change of the AD brain. It starts temporally due to hippocampal neurodegeneration, with further progression to the parietal lobe, all resulting in the impairment of episodic and subsequently of long-term memory, as the dominant clinical AD features (29-32).

According to the literature data, the amyloid cascade and neuroinflammation, deserve special attention among all other AD hypotheses, and thus, they will be more discussed below. The first one represents one of the unambiguously demonstrated, central events in AD – pathological deposition of amyloid proteins. The second one seems to offer comprehensiveness and a more successful explanation of early events in AD and disease dynamics.

1.2.1. Amyloid hypothesis (AH) – decades-long dominance justified

The amyloid cascade hypothesis has dominated for a long time as an explanation for the pathogenesis of AD, placing the Aβ accumulation as a crucial pathological event and driver of the pathophysiological process, finally responsible for the cognitive deterioration of individuals suffering from AD.

First discovered in patients with cerebrovascular disease (53), disordered amyloid metabolism was established as the initial pathophysiological cascade in AD, after a series of genetic and experimental discoveries in the early onset, familial disease forms (EoAD) in the 1980s (38,39). Relationship of the final pathological product - Aβ, with various mutations, detected by sequencing - mainly in *APP*, *PSEN1* and *PSEN2* genes (38,54,55) is considered to be the initiating factor in dysregulated amyloid processing and formation of Aβ deposits (31,32,33,55). It has to be emphasized that the molecular findings in EoAD were also presented as the most probable mechanism in LoAD, since neuropathological and clinical features in both disease forms were known to be identical (31,32,39).

In short, AH proposes amyloid-β protein precursor (AβPP) - widely expressed transmembrane glycoprotein in the human body, as the starting point of the pathological cascade, resulting in Aβ accumulation (29-34). In the central nervous system (CNS), this protein is normally processed through two different pathways: non - amyloidogenic and amyloidogenic (56, 57). Both of the pathways represent a two-stage enzymatic lysis cascade, but the enzyme which cleaves APP in the first step is different in each pathway (57,58). Amyloidogenic, labeled as a pathogenic pathway, produces several Aβ types, including the two most common fragments consisting of 40-42 amino acids (Aβ 40/42). However, it is also active in normal conditions, and Aβ production has been also reported in healthy people (56,57). However, Aβ generation and clearance in non-pathological circumstances are balanced. It is considered that only a small fraction of AβPP has been degraded through the amyloidogenic pathway, resulting in the production of Aβ, which remains soluble (55-58). In the progressive AD course, on the other hand, increased dynamics of amyloidogenic processing and/or facilitated APP enzymatic lysis, fostered by various mutations in AD-related genes, results in overproduction of Aβ, which, in such circumstances, self-aggregates (52,59,60). The longer the Aβ fragments, the greater the probability of their assembling, and so the longest one - Aβ42, mostly forms oligomers, amyloid fibrils and eventually plaques, known to represent the neuropathological, amyloid AD substrate (29,31,34,52). It is interesting to note that in contrast to earlier findings, recent research points to higher toxicity of Aβ oligomers, compared to fibrils and plaques, inducing more severe neurodegeneration . More complex forms seem to induce aggregation of Aβ monomers, contributing to the expansion of the neuropathologic area in the brain but do not exert toxicity (61). Interestingly, other research indicates that not only insoluble but also soluble Aβ forms are toxic (62). In general, the hypothesis places the accumulation of Aβ in the central position, considering that its toxicity certainly represents the necessary and main cause of all, various downstream events, finally leading to a synaptic and neuronal injury and death (51,52,55). Namely, it has been postulated for a long time, that hyperphosphorylation of tau protein, oxidative stress, neuroinflammation, vascular damage, and finally cognitive decline, are all Aβ-induced events (33,34,51,52,63), but precise mechanisms or their temporal ordering, are missing. Therefore, the research which followed the formulation of AH gave rise to several

mechanistic explanations, many of which are arguing for opposing conclusions. As an example, the dual hypothesis proposes that tau hyperphosphorylation is not the consequent but rather the process parallel to Aβ deposition, induced by the same event, but both are linked through an additional mechanism (48). The other data have weakened the clinical significance of the AH, like those coming from the first studies investigating the efficacy of Aβ clearance from the AD human brain. Some of them have failed to demonstrate improved cognitive status after the reduction of amyloid load (64). Hence, it is still questioned which are the downstream processes of Aβ accumulation, whether some have mutual influences, and what actually represents the starting point of AD pathophysiology. Finally, it has become clear that this single theory cannot explain the complex pathophysiological context of AD. Instead, several biological pathways have been proposed as the leading or complementary to AD amyloid theory: transport and metabolism of lipids, intracellular vesicular trafficking, immuno-inflammatory response, apoptosis, synaptic failure, oxidative stress, calcium metabolism, iron homeostasis, and mitochondrial dysfunction (29,32,35,65,66).

Eventually, despite the evident ambiguities of AH, the presence of amyloid deposits, as the pathomorphological substrate of AD, has never been disproved and understandably, the hypothesis persists. Both, clinical-pathological studies as well as current AD biomarkers studies, indicate that amyloid accumulation represents a very early event, which probably occurs even decades before the clinical manifestation of the disease (44). Moreover, the first biomarkers identified, are those that detect Aβ pathological change in the brain (30). Therefore, decades after its formulation, the researchers are still focused on the elucidation of the amyloid cascade, as well as on the investigation of therapeutic solutions based on it. This is evidenced by the involvement of almost 50% of agents targeting Aβ, among 120 treatments for AD, that were either in phase 2 or 3 in clinical trials, in 2021 (67). Furthermore, after numerous challenges to show the effectiveness of such therapeutic modalities (64), the clinical use of two amyloid-reducing agents has been finally approved by Food and Drug Administration (FDA) (68). Therefore, the translational value of AH has been proved, justifying its long-standing existence. Although this theory is experiencing and will

experience modifications, it now appears that the AH will probably remain the pathophysiological backbone of AD.

1.2.2. Neuroinflammation - the potential for a holistic explanation of Alzheimer's disease

Among biological pathways which have been proposed as the leading AD mechanisms, inflammation has emerged as a substantial driver of AD (66). The importance of inflammation in the neurodegenerative processes of AD was observed for the first time 30 years ago, in a study that reported the positive effects of chronic anti-inflammatory therapy, which caused a decrease in the incidence and progression of AD (69). Epidemiological studies have also shown positive correlations between dementia and data on previous systemic infections (70). A number of studies that confirmed these initial observations followed. An interesting experimental study on the primate model has demonstrated that the injection of Aβ alone into the brain did not result in amyloid deposition; however, co-injection of Aβ and lipopolysaccharide, which primes proinflammatory innate immune response, led to the pathological accumulation of amyloid proteins (71). Clinical trials have also provided supportive evidence. Increased levels of inflammatory markers in AD patients have been reported (72,73), and interestingly, even the association between cognitive impairment and blood level of C-reactive protein – a routine marker of inflammation, has been demonstrated in a large, diverse sample of adults (74).

Further research offers more concrete, pathophysiological explanations of the role of neuroinflammation in AD. The association between the genes that confer increased risk for AD and innate immune function has been shown (65,75), and this has contributed to a deeper understanding of the importance of neuroinflammation in AD pathogenesis. Interestingly, an incidence of LoAD and polymorphisms of genes that encode key proteins of the innate immune response were found to be in correlation (76,77). It has been further demonstrated that the activation of the CNS immune system represented by microglia, drives the neuroinflammation process, playing an important role in AD pathogenesis (78,79). Namely, as key immune brain cells, microglia recognize amyloid deposits and subsequently respond through the initiation of inflammation (80,81). This is followed by the activation of pro-

inflammatory cytokines as well as the release of reactive oxygen species (ROS) (82). Accordingly, studies have shown that proinflammatory cytokines and chemokines significantly increase in the brain of AD patients (72,73). Moreover, inflammatory mediators have been highly expressed in these individuals, particularly in the area of Aβ peptides and neurofibrillary tangles (83). Even more interesting findings point to essential interconnection and mutual influence between amyloid cascade and neuroinflammatory process in AD. Namely, it has been demonstrated that the production of Aβ peptide as well as its processing, have been impacted by the specific, activated cytokines – interleukin - 18 (IL - 18), or a combination of interferon-gamma (IFN - γ) and tumor necrosis factor-alpha (TNF - α) (84,85). Eventually, studies that have explored functional and structural changes in the AD brain have reached interesting and important conclusions, that help to completely understand and judge the translational significance of neuroinflammation. One of them has demonstrated an inverse correlation of microglial activation with hippocampal volume and metabolism of glucose in AD patients (86,87). The other has found that cognitive performance was negatively correlated with microglial activation (88,89). Collectively, this evidence clearly implies that neuropathological brain changes progressed, as the activation of a major carrier of neuroinflammation increased.

Neuroinflammation theory offers the pathophysiological context of events or individual aspects of AD that we could not adequately understand and explains the driving mechanism for the disease. Recent evidence indicates that neuroinflammation may precede or even cause the generation of amyloid and tau cascades (90,91,92), placing it, not only at the center but also at the beginning of the AD pathophysiological process. Moreover, neuroinflammatory events further interfere with amyloid and tau pathways and determine early AD progression (84,85,91,92,93). Finally, the role of microglia might be highly important in the process of synaptic dysfunction and loss in AD (94), although precise mechanisms are not clear. Therefore, immunity and neuroinflammation might be key carriers of both, the increased risk for AD as well as of its pathogenetic process, from the initial point until neuronal death, providing a comprehensive and fundamental explanation of AD pathophysiological context.

1.3. Clinical concept and biomarkers of Alzheimer's disease

1.3.1. Nature of Alzheimer's disease

AD represents the most common cause of dementia among the older age population and it is characterized by insidious onset, leading to progressive memory loss and cognitive deterioration (29,30,32). Although age is considered a main risk factor for AD (1,2,3,29,30,95), the disease may also occur earlier in the lifetime, mainly in individuals who have familial gene mutations. Familial gene mutations are present in 5% of diagnosed cases, while 95% of AD patients represent sporadic cases (2,29,31,32,39). Nevertheless, in terms of AD classification, this type of traditional division of the disease into familial and sporadic cases has been overrated, since sporadic cases partly occur at an earlier age and familial forms may appear in the elderly. Therefore, the temporal determinant of the disease onset was the most consistent criterion for the classification of AD, so there are currently two main categories: EoAD < 65 years and LoAD > 65 years (29,30,96). In both forms, specific pathological characteristics of the disease (AD - P) cause the whole spectrum of clinical manifestations (AD - C), which typically include memory impairment regarding the consolidation of information, but relatively spared recall, incremental loss of cognitive functions, involving the language, visuospatial and executive domains (29,31,96). Neuropsychiatric and sleep changes are often concomitant disorders. Moreover, anxiety, depression, or aggression also occur, and sometimes may be noticed early in the disease course (29), leading to diagnostic dilemmas. Furthermore, significant pathophysiological diversity has been detected in established LoAD cases. Amyloid plaques and tau tangles are sometimes mixed with deposits of alfa-synuclein or TDP - *43*, as well as with alterations of hippocampus or microvasculature (97,98), causing variable clinical presentations and uncertainties in clinical diagnostic approach (99). Moreover, it was observed that, the higher the age in diagnosed AD cases, the greater the overlap with neuropathological changes in cognitively intact old individuals (100,101). Polygenic nature of both forms – EoAD and LoAD make the disease even more complex (29,38,39,96,102). Namely, typical clinical syndrome or AD phenotype can be caused by different genetic alterations, that is, distinct

mutations or polymorphisms at different positions in the same gene leading to the same pathophysiological consequences and disease presentation (102). On the other hand, different mutations within the same gene may also result in different settings of AD clinical manifestations (103,104). In accordance with such a genetic, pathophysiological and clinical heterogeneity, the concept of AD detection and evaluation itself, evolved significantly over time, reflecting the complexity of this disease, too.

Namely, after a long period of the purely clinical approach, when AD diagnosis was considered only in those who manifested specific cognitive impairment (CI) and its confirmation was possible only pathohistologically - post mortem, a significant conceptual shift has happened in the last decade. First of all, it is now clear that AD is a dynamic, continuous, and progressive process, from asymptomatic disease to dementia (30,105). At the beginning of the AD continuum, around 30% of individuals are estimated to have some level of AD - P, sometimes even significant brain changes, but no apparent symptoms (106). Thus, it does not mean, that once occurred, pathological brain changes due to AD cause dementia and AD diagnosis is not anymore solely related to the presence of symptoms. In fact, neurodegeneration evolves gradually over time, and when clinically manifested, the disease had previously been developing for probably a couple of decades (29,30,44,45). One of the explanations of such a nature of AD has been based on the growing evidence on the role of neuroinflammation, as the leading pathophysiological theory of AD, as described above. Namely, it is well known that an aging organism is susceptible to the development of low-grade inflammation (inflammaging), which represents a risk factor for both, morbidity and mortality (107). Therefore, similarly to other diseases, the concepts based on the chronic development of inflammation associated with aging, such as late-onset cancer, type II diabetes, and cardiovascular disease, LoAD may be understood as an age-related chronic disease (107,108,109). That could clarify the gradual accumulation of pathological changes in the AD brain and the dynamic nature of this disease, with a long preclinical phase. Certainly, the explanation for the preserved cognition despite the development of AD - P in some people, could be based on the wide variation in baseline neurocognitive abilities (among individuals in one population). The better the baseline, the better the ability of the

individual to compensate pathological context that has occurred. This actually refers to the concept of the reserve in the AD field and cognitive aging in general, represented by the two entities defined by Barulli and Stern (110) and Stern et al. (111). Brain reserve supports cognition in a quantitative manner, that is, relies on individual structural capacity and integrity of the brain, involving a number of neurons and synapses. On the other hand, cognitive reserve (CR) is a spectrum that we can build throughout life and increase its capacity through cognitive and social experiences or physical activity. More precisely, it includes education level, cognitively stimulating activities, occupational complexity, quotient of intelligence, as well as bilingualism and socioeconomic status, as the most important aspects (110-113). It is reported that the equal AD-P level will be later manifested and clinically diagnosed as AD, in individuals with higher brain and/or CR, compared to low--reserve individuals (114,115). Evidence coming from the other studies further demonstrate the power of individual neurocognitive resources, reporting an increased likelihood of reversibility of MCI to normal cognition in individuals with higher education and better global cognition (116,117).

1.3.2. Significance of biomarkers of Alzheimer's disease

The conceptual change of AD was strongly supported by the biological approach. In other words, described the discovery of the heterogeneous and dynamic nature of AD was made possible by the progress of research in the field of biomarkers. With a previous, pure clinical approach, only the manifested disease could be diagnosed, that is, during the phase which is currently known to be only a culmination of the long-term pathological process. However, aided by the biomarkers, we are able to define the presence of AD in an individual, in the absence of clinical manifestation (30). The biological concept and definition of AD emphasize that clinical presentation - cognitive impairment, represents only a symptom or sign of the disease, that is, a reflection of one or more neuropathological constructs, whose nature may be accurately assessed only biologically - by biomarkers (30). Therefore, biological markers helped the AD field of research to essentially move forward.

Current AD biomarkers are most easily divided according to the methodological approach for their detection: biochemical, cerebrospinal fluid (CSF) – derived, and imaging-derived biomarkers (118,119). On the other side, considering the pathological processes detected by biomarkers, they are classified as those that measure the accumulation of Aβ, neurofibrillary tau deposits, and neuronal injury or neurodegeneration (120). Currently, there are two biomarkers of extracellular Aβ accumulation – low CSF Aβ (121,122,123) and abnormal tracer retention on amyloid positron emission tomography (PET) imaging (124,125). Biomarkers of tau pathology are represented with elevated CSF P - tau and tau PET ligand binding in the cortex (126,127). Neuronal injury or neurodegeneration is manifested as hypometabolism, detected by fluorodeoxyglucose (FDG) PET or atrophy identified by magnetic resonance imaging (MRI), in brain regions typical for AD (128), and can also be measured by total tau (T-tau) level in CSF (129). The validity of these biomarkers as AD preclinical "biological signature" has been extensively studied, and has been dominantly focused on the preclinical existence of amyloid type of AD pathology. In presymptomatic autosomal dominant mutation carriers, and also in those who are asymptomatic at risk for clinical AD, it has been shown that amyloid pathology detected by altered CSF levels or PET imaging, occurs up to 20 years prior the clinical AD manifestation (44,130,131). In addition, amyloid changes identified postmortem in cognitively normal individuals are in agreement with these findings (132). Although tau pathological changes have been also confirmed to occur more than 10 years before clinical AD presentation (45), it is currently established that detection of amyloid AD neuropathological substrate only is necessary to define that brain pathological change is of Alzheimer's type (30). Actually, in order to adequately define AD stages and understand the biological importance of the presence of each type of currently known pathological AD change, or their combination, an ATN system of classification was established by the National Institute of Aging – Alzheimer's Association (NIA – AA) group (Table 1). With each of the three letters indicated, this scheme identifies a specific pathological process – amyloid („A"), tau pathology („T") and neurodegeneration („N"), detected either by CSF or imaging biomarkers, enabling the creation of specific ATN profile for each individual (30). According to this official and widely accepted classification, the positive amyloid biomarker is enough to consider that an individual belongs to the AD

continuum. Furthermore, tau positivity together with the presence of amyloid AD change determines that someone has AD. Although non-specific for AD, neurodegeneration is in strong correlation with symptoms (133), thus, detected together with amyloid change, it contributes to better prediction of future cognitive decline, but it could also indicate the possibility of simultaneous, non-AD pathologic change (30).

Although the ATN individual profile biologically determines the AD continuum, it does not necessarily indicate the clinical severity of the disease. On the other hand, unlike the neuropathological substrate detected by biomarkers, clinical AD presentation is non–specific and thus, does not have the capacity to define the disease. Actually, cognitive impairment, even multidomain amnestic dementia phenotype – considered typical for AD, represents clinical consequence not only of AD pathology but also of other diseases (5,6,134,135). It means that it is a syndrome, whose etiology should be biologically confirmed. In addition, visuospatial, executive, or language disorders as non-amnestic presentations, may also be caused by AD (136,137). Furthermore, in the older age population, AD neuropathologic changes are often present without signs or symptoms (132). Therefore, current biomarkers have enabled substantial improvement in understanding AD, from clinical-pathological to biological construct. They have an invaluable role in increasing diagnostic certainty in the evaluation of MCI and dementia syndrome (138,139,140), high importance in the evaluation of those with oligosymptomatic clinical manifestations (141), and a unique capability to identify individuals with ongoing AD-P, in the absence of manifested cognitive impairment (142,143). However, despite the objective limitation for neurologists to declare that AD syndrome is etiologically AD, without biological confirmation, current AD biomarkers are not widely available for routine clinical practice. That is probably the main reason for the flexibility of official diagnostic criteria, which do not oblige clinicians to include information on current biomarkers in the process of diagnostic decisions related to AD (30). The biological concept of the disease presented above is limited only to research (30), which will be more discussed below. With the growing knowledge about the complex genetic and pathophysiological context of AD, as well as other overlapping syndromes and other neurodegenerative diseases occurring in the population of similar age, a purely clinical

approach to AD demonstrated modest diagnostic potential (143-154). It has been shown that 25%-30% of patients evaluated at specialized dementia clinics were clinically misdiagnosed as AD dementia (144,145,146). At the primary healthcare level, even 50% - 70% of symptomatic AD cases did not receive correct diagnoses or remained unrecognized (147,148,149,150). Among those who underwent an autopsy, around 10% - 30% of AD dementia cases clinically diagnosed by experts, did not have AD neuropathologic changes (149,151). The neurodegeneration found was not associated with amyloid pathology, but rather with mid-life risk factors, such as obesity, smoking, diabetes, or cardiovascular diseases (152,153,154). Therefore, although the routine clinical approach is mainly based on cognitive assessment, there is evidence that cognitive symptoms are not a completely reliable way to define AD. Finally, it has been reported that clinical evaluation indicated AD neuropathological alterations with sensitivity and specificity of only 81% and 73%, respectively (143,144,146).

Table 1. AD biomarker profiles and categories

AT(N) profiles	Biomarker category	
A-T-(N)-	Normal AD biomarkers	
A+T-(N)-	Alzheimer's pathologic change	AD continuum
A+T+(N)-	Alzheimer's disease	AD continuum
A+T+(N)+	Alzheimer's disease	AD continuum
A+T-(N)+	Alzheimer's and concomitant suspected non - Alzheimer's pathologic change	AD continuum
A-T+(N)-	Non-AD pathologic change	
A-T-(N)+	Non-AD pathologic change	
A-T+(N)+	Non-AD pathologic change	

Every individual can be placed into one of the three general biomarker "categories" based on biomarker profiles: those with normal AD biomarkers (no color), those with non-AD

pathologic change (dark grey), and those who are in the Alzheimer's continuum (light grey). The term "Alzheimer's continuum" is an umbrella term that denotes either Alzheimer's pathologic change or AD. Source: modified from *Jack CR et al., 2018* (30).

1.3.3. Cognitive continuum of Alzheimer's disease

Even in the cases of confirmed pathology of AD type, the same level of AD - P may be manifested in each individual at different clinical AD stages, probably due to different individual neurocognitive reserve, presence of risk factors, or simply heterogeneity of the genetic and pathophysiological aspects of the disease. Therefore, the cognitive performance and biological continuum are not necessarily in correlation and in fact, the cognitive presentation of AD represents the continuum itself, which is assessed by clinical evaluation and neuropsychological cognitive instruments (30). It is still challenging to clinically determine each AD stage, especially when it comes to earlier phases in the disease course. Events of the onset as well as points of the disease transition through stages (138) have not yet been properly defined and standardized. However, according to the official diagnostic and research criteria, there are three main categories that indicate cognitive status or severity of cognitive impairment: **cognitively unimpaired (CU)**, MCI and dementia (30,138-140,142). Clinical assessment and neuropsychological-cognitive evaluation for CU individuals are expected to be in the standardized range. However, sometimes it may be impaired according to the general population norm, but expected a for particular person, taking into account age and education (138-140). On the other hand, having in mind the biological AD continuum, CU - labeled cases in the cognitive staging scheme, may actually belong to the preclinical AD stage, but such a conclusion has to be based only on evidence of present AD biomarkers (30). It is worth noting that subtle clinical manifestations, prior to the objective evidence, are also currently recognized as a useful tool for the early identification of AD. Namely, criteria for identification of **subjective cognitive decline (SCD)** due to AD are structured (155), proposed by the Subjective Cognitive Decline Initiative (SCD - I), and widely accepted. However, self-perceived cognitive decline is actually one aspect of the SCD structured concept, which also includes additional anamnestic

and hetero-anamnestic data, as well as evidence on the presence of ApoE ε4 genotype and AD biomarkers, in order to confirm that SCD detected is due to ongoing AD pathology (155). Therefore, wide, routine application of such a defined concept is limited. In addition, there are inconsistent and heterogeneous findings regarding the psycho-social and intellectual aspects of the patient, as well as interpersonal relationships during the assessment, and thus its clinical utility (156,157). Nevertheless, purely clinical evaluation of subjective cognitive status through an open - type questions, which is supported by the SCD framework (155), certainly represents a helpful concept that moved clinical routine toward better early identification of possible development of AD syndrome.

The earliest AD stage available for objective assessment by currently accepted diagnostic tools (30,139) is MCI. **MCI concept**, introduced by Petersen et al. more than 30 years ago (158), has been often inconsistently used in research and clinical settings, with different terminology, leading to unusable or confusing data. However, current criteria were established in 2011 and refined in 2018 for research purposes, are clear and well - standardized, and thus successfully improved both, clinical and research practice (30,138-140). MCI syndrome is such a level of cognitive decline, which is generally between physiological aging and dementia and exists in many forms. According to many reports, as well as the official guidelines, if AD is probable underlying pathology, MCI syndrome is predominantly of amnestic type (139). It means that the most prominent clinical observation and neuropsychological finding is subjective and objective impairment of episodic memory, that is, the ability to learn and retain new information. General cognitive function is mostly intact in individuals with MCI, but such cognitive status may mildly impact their complex daily life activities (30). Although it is widely accepted that people with amnestic MCI syndrome are more likely to develop AD and progress to dementia (139), it should be emphasized that other MCI forms, including multiple-domain and single non-memory-domain types (159), may also be caused by AD. Notably, considered an early phase in the disease trajectory available for standardized clinical identification, the MCI stage also represents valuable and suitable ground for the investigation of early AD pathological processes. More importantly, the available data imply the possibility of effectively slowing down the disease process precisely at the MCI stage, since MCI could progress to dementia

due to AD, but also remain stable over time (160). The reversion of MCI to a normal level of cognition has been reported, too (116,117). On the other hand, unlike MCI patients, those in asymptomatic stages of the disease are still hardly available for recruitment. Therefore, many research efforts are currently directed towards the identification of biomarkers of the MCI stage, which will hopefully identify key early processes in AD and lead to the facilitated detection of the preclinical stage of the disease.

Successful clinical assessment of progression from MCI to **dementia syndrome** is based on continuous or repeated evaluation of the patient's cognition and abilities to perform instrumental activities of daily living (140). Generally, the transition point may be established when new disabilities are detected, in comparison to a previous state of cognition and functions. Although diagnostic guidelines are established and clear, each patient should be independently assessed by the skilled clinician, in the context of age, education, general intellectual capacities and previous clinical state (30,140). Together with the history of worsening, detected cognitive or behavioral impairment in at least two domains and impaired task handling, increases level of certainty in AD diagnosis. Multidomain amnestic dementia is considered a typical clinical presentation of AD, however, this phenotype could be also caused by other diseases. On the other hand, the underlying cause of non-amnestic presentations, executive, language or visuospatial, may also be due to AD. Impaired status of cognition and functions of daily living generally impacts the independence of dementia patients (30). AD dementia syndrome is subdivided into **mild, moderate and severe** stages, and so the level of disability may vary accordingly (140). It is actually very important to keep in mind such cognitive continuum of dementia syndrome itself, because, detection of its early phase may be still considered as timely, in the context of therapeutic possibilities. In agreement with this, the mild dementia stage is often unified with MCI in the **early AD (EAD)** in many studies (68,161,162,163,164,165). Namely, this concept is especially used in the last few years, in light of the criteria for therapy with monoclonal antibody - aducanumab, which was the first amyloid–reducing therapy approved (68). Unlike the last two dementia stages, characterized by advanced disabilities, clinically determined EAD patients are often suitable for this treatment, according to the inclusion criteria (68). However, the presence of amyloid pathology in these patients has to be confirmed by amyloid

PET (68). After all, classifications should exactly have a practical purpose, determining prognostic and therapeutic possibilities for AD patients.

Finally, the presence of Alzheimer's clinical syndrome, which applies to both, MCI and dementia patients, does not automatically imply the presence of AD neuropathological changes and thus, does not confirm the presence of AD itself (5,6,30,134,135). In addition, the absence of the prototypical AD syndrome does not imply the absence of AD pathology (30,97,98,99,136,137,159). Although it represents a diagnostic tool that is widely used in routine clinical practice, it has been shown that the capacity of cognitive staging is actually limited to a proper definition of the current clinical state and determination of disease severity.

The evolution of the discovery and knowledge regarding AD has led us to conclude that the clinical and biological aspects of the disease cannot be equalized, and therefore, it still leaves us on unsafe clinical ground. In other words, the paradox of the existence of AD biomarkers is that they cannot be widely used in routine clinical practice and help in solving clinical dilemmas. Therefore, even at the moment when the diagnosis is biologically already determined, its confirmation cannot be reached in the absence of evidence on biomarkers. Only with the further development of clinical presentation, we can slowly raise the level of certainty that a particular syndrome is etiologically AD. The most unfortunate circumstance is, that meanwhile, we deprive patients of the possibility of timely diagnosis and application of available disease-modifying therapeutic methods. Thus, further evolution of the research and knowledge is expected to enable not only simple biological confirmation of the justified clinical assumption of AD diagnosis but also to move us a step further – to enable early diagnosis of clinically unmanifested disease. Therefore, the need for new biomarkers is undisputable and urgent.

1.3.4. Potentially new biomarkers of Alzheimer's disease

It is promising that research is already progressing regarding the development of new biomarkers. The dominant direction represents the investigation of blood-based biomarkers, which are expected to have the capacity to overcome the main disadvantages of the current AD biomarkers. Easily and widely accessible, they could certainly open the screening possibilities and thus serve to reach the goal of early AD detection. In addition, lumbar puncture is an invasive procedure, while PET scans are expensive and often not available, which greatly limits the population-wide applicability of current biomarkers. Collection and processing of blood-based biomarkers would be minimally invasive and more affordable, allowing for more frequent sampling, for the clinical as well as for the research - related follow – up(s). So far, the research has identified several new, potential biomarkers, which could reflect brain amyloid and tau positivity and neurodegeneration, through the detection of their blood levels: Aß, tau protein, neurofilament light (NFL) and glial fibrillary acidic protein (GFAP) (166,167,168).

Reduction of blood $Aß_{42}/Aß_{40}$ ratio, similarly as in CSF, indicates progressive depositing of $Aß_{42}$ in brain amyloid plaques. Importantly, such a change in this potential AD biomarker can be found before the first disease manifestation. Moreover, $Aß_{42}/Aß_{40}$ ratio is altered to its full extent at that moment of detection (169). These advantages, contribute to such a high accuracy of the test in CU individuals, as it is observed in those with clinically manifested AD. In addition, the plasma amyloid status correlates well with the future positivity of the PET amyloid test (170). However, only small changes have been observed between Aß - positive and control individuals, which is the limiting factor for the application of $Aß_{42}/Aß_{40}$ blood ratio as an AD biomarker (171,172). Namely, brain amyloid pathology does not affect extracerebral amyloid production (173), which probably contributes to the smaller change of plasma $Aß_{42}/Aß_{40}$ in AD. This could be at least a part of the reason why the standardization of cutoff values for plasma $Aß_{42}/Aß_{40}$ has not yet been reached worldwide (169). In addition, although several methods for its detection have been already developed, there is no clear consensus on which one should be used as a standard (174).

Plasma p-tau, which has been related not only to the tau tangles but also to the density of Aß (175), demonstrated high specificity for AD. The results from the large clinical studies indicate a strong capacity of p-tau to identify AD among other neurodegenerative diseases (175,176). Currently developed assays can determine variants of tau protein, phosphorylated at the following N-terminal amino acids: 181, 217, and 231 (p-tau 181, p-tau217 and p-tau231). Increment in the plasma level of these p-tau isoforms is even specific for AD compared to other tauopathies (177). Moreover, each variant seems to have different advantages, and all p-tau isoforms may complement each other as AD biomarkers. While the p-tau231 level has been shown to change earlier than other variants in the disease course, p-tau217 has demonstrated the best diagnostic performance (178,179). Importantly, changes in levels of p-tau217 and -181 have been shown to be able to determine the potential for conversion from MCI to dementia due to AD, in 2-6 years, following the test (175,180,181). However, fully automated assays for different p-tau variants are still under development (174).

The same as the previous two biomarkers, NFL has been initially isolated from CSF and used as an indicator of neuronal injury. It is the most abundant among neurofilament proteins in neuritic plaques, which are the products of the dystrophic neuronal process caused by neurodegenerative disease (182). Higher levels of NFL have been detected in AD patients, however, compared to other neurodegenerative diseases, this increase is rather modest (183,184). Especially delayed is NFL blood detection in sporadic AD cases - the earliest known detection was during the MCI stage of AD (185). Additionally, the increase in NFL level not only reflects neurodegeneration but also strongly correlates with age (183,186), which implies the lack of specificity. These are the main limiting aspects regarding the diagnostic performance of blood NFL levels in AD. However, NFL level promptly changes with the dynamics of neurodegeneration and thus, might usefully complement other biomarkers, considering that the rate of neurodegeneration is closely related to the progression of AD clinical manifestations (185). Several other, axonal and synaptic proteins, have been investigated and proposed to indicate neurodegeneration, but have not demonstrated clinical usefulness (187).

GFAP is a glial biomarker strongly expressed in astrocytes (188,189). Considering that the glial activation is mainly induced by Aß deposition, blood levels of GFAP are considered a reliable reflection of the Aß accumulation process (188), which is the hallmark of AD. Moreover, GFAP has shown a potential to predict subsequent AD stages in CU and MCI individuals (189). Although it cannot be considered AD-specific, GFAP plasma levels are significantly less changed in non-AD neurodegenerative diseases, with the exception of progranulin mutation-related frontotemporal dementia (190). On the other hand, it has been reported that traumatic and cerebrovascular brain injury may also cause changes in GFAP levels (191,192). In general, more research is still needed to examine clinical confounders, other potential biological impacts on GFAP level, its diagnostic performance in different clinical contexts, as well as to better understand its relationship with pathological mechanisms of AD (174).

According to the recommendations of the Alzheimer's Association, published in 2022, there is a need for head-to-head, longitudinal and real-world studies in diverse populations, as well as for adequate laboratory assays and established universal cut-off points, in order to confirm the applicability of each of these biomarkers and their combination in various clinical scenarios (174).

1.4. Current status of therapeutic modalities and preventive strategies for Alzheimer's disease

Research dynamics and efforts focused on therapeutic solutions for AD, are in a high discrepancy with current possibilities to treat this, still incurable disease. Symptomatic modalities that have been in use since 20-30 years ago, still represent the only available treatments, in routine clinical practice. The first therapeutic choice showed to improve cognitive status in early dementia stages represents agents which inhibit the enzyme acetylcholinesterase: rivastigmine, donepezil, and galantamine (29), leading to the increased amount of acetylcholine (Ach) in the synaptic cleft (29,193). Namely, it has been shown that cholinergic transmission is negatively influenced by neuronal injury in AD, and that hippocampal and cortical processing of information may be partly compromised due to Ach deficiency (194). Recent findings show that in addition to inhibiting acetylcholinesterase, rivastigmine can also shift the APP cleavage process towards the protective, non-amyloidogenic pathway (195). This evidence implies the possible disease-modifying capacity of rivastigmine and supports data on close interaction between the cholinergic system and Aβ (196). N-methyl-D-aspartate (NMDA), a glutamate receptor, represents the other target of approved anti-AD agents, such as memantine, indicated for moderate to severe AD (29,193). This NMDA competitive antagonist has been shown to improve cognition, as well as behavioral and psychological symptoms of dementia, which are often present in AD patients (29,197). These positive effects are based on the finding that glutamate-mediated toxicity and the following calcium overload, which lead to mitochondrial dysfunction and high levels of ROS, finally result in neuronal death in AD brain (198).

After the approval of memantine in 2003, there were no new drugs on the market for 18 years. Meanwhile, hundreds of new medications have entered clinical trials, the majority of them focused on disease-modifying mechanisms, based on AH and tau biology (67). At the time the first immunotherapy was approved in 2021 (68), there were around 130 agents for AD in phases 2 or 3, with the disease-modifying agents in more than 70% of clinical trials (67). Aducanumab, an immunotherapy agent, is expected to influence disease progression, and not only alleviate symptoms since it has a mechanism of action based on the reduction of amyloid (68). This therapy was initially challenged, because it did not demonstrate

improvement in cognition and functional abilities of patients, after removal of Aβ peptide deposits (64). However, these failures lead to conclusion, that this kind of therapy, which should modify the disease course, had to be tested earlier in the pathophysiological AD continuum (199). After implementing such a strategy, success is evident. Aducanumab is a recombinant monoclonal antibody, whose generation has been based on antibody genes from the circulating lymphocytes of healthy and cognitively normal elderly subjects (200). Reduction of Aβ peptide is achieved by acting on its N-terminus (200). The use of this therapy is indicated in patients with early AD – those with MCI due to AD and those with mild AD dementia. However, when selecting patients who are eligible for therapy, after the cognitive staging there are additional, strict and precise criteria. Namely, just like it was done in the aducanumab trial, the presence of an amyloid target has to be demonstrated by positive amyloid PET (68). Such an approach is strongly supported by the fact that up to 40% of patients diagnosed clinically, did not have amyloid pathology when examined by amyloid imaging (201). Furthermore, mandatory MRI and a whole range of clinical, neuropsychological and laboratory criteria, that must be met for the use of aducanumab, require skilled, expert clinicians (68). In addition, aducanumab treatment may be accompanied by a higher incidence of amyloid-related imaging abnormalities (ARIA), especially the occurrence of brain effusion or hemorrhage. Therefore, the examination by MRI has been recommended before beginning the therapy, during its titration, or in case of symptoms that might imply the occurrence of ARIA (68). Application of this type of therapy requires expert staff and advanced infrastructure, which makes its widespread use difficult to achieve. Eventually, the entry of aducanumab to the American market was followed by the approval of the second monoclonal antibody – lecanemab, at the beginning of the year 2023 (202). Compared to aducanumab, it targets more complex Aβ fragments – protofibrils, which also seem to promote the assembling of the free monomers and contribute to the expansion of Aβ plaques (202,203). For this reason, there are considerations, that lecanemab might reach more significant clinical usefulness than aducanumab. Both agents are currently available only in the United States, however, there are indications that this will also happen soon in Europe. Other treatments targeting amyloid cascade, currently under investigation, are aimed at the reduction of AβPP generation, inhibition of its cleavage, enhancement of its

metabolism through non-amyloidogenic pathway, as well as those preventing Aβ aggregation (204).

Initial challenges in the process of testing amyloid reducing therapy described, have fostered the development of strategies targeting tau protein. These agents are still in clinical trials (205), but on the rise, in the last years. They are mainly focused on the prevention of pathological hyperphosphorylation of tau protein (205). However, recent evidence on extracellular tau accumulation (35) has encouraged strategies aimed at targeting and preventing its interneuronal spread (36,37), and finally, tau intracellular deposition.

No doubt that the current status of therapeutic possibilities for AD, which still does not include causal treatment, is a reflection and/or confirmation, of many unknowns regarding the pathophysiology of this disease. However, a recent, better understanding of AD biology and disease course - with a long preclinical phase, has imposed a need to manage early neuropathological changes, known to compromise normal biological processes, before cognitive impairment occurs. Although the new, amyloid–reducing therapy is intended for oligosymptomatic patients, it certainly reflects such attempts. Additionally, slowing of cognitive decline has been demonstrated to positively correlate with non - pharmacological treatments, which include improvements in nutrition and physical activity as well as cognitive training (206). However, there are contradictory findings regarding their effectiveness in the stages of manifested cognitive impairment (208,209), and no doubt that these strategies are much more successful when applied as preventive (206,207,208,209,210). Namely, there is strong evidence that the lower levels of formal education and intellectual challenges, physical and leisure activities, as well as poor quality of the social network, are associated with increased risk of cognitive decline and AD (211,212). An important area of other modifiable risk factors for AD represents treatable diseases that usually occur in midlife, such as hypertension, obesity, diabetes, hypercholesterolemia, and dyslipidemia (29,32,213,214). It has been well demonstrated that hypertension and cardiovascular damage, may directly compromise the complex vascular support of the brain, contributing to the negative effect of AD-P with aging (142,213,215). Lipid homeostasis, especially cholesterol, is highly involved in brain functions and its

imbalance has been strongly related to the occurrence of AD (216), having a role in the amyloidogenic pathway as well as in the process of tau hyperphosphorylation (213,217,218). Dyslipidemias are interrelated with obesity and brain insulin resistance in the development of AD (219). According to the literature, the last one is a particularly important factor in the molecular processes of deposition of amyloid and neurofibrillary tangles (220). Thus, in recent works, AD has been even labeled as type III diabetes (220). When investigated in manifested disease, diabetes was demonstrated to increase the risk of conversion of any type of MCI to dementia (221). Mental imbalance, including stress, anxiety and depression, has been clearly linked with an increased risk of AD (222,223). Interestingly, although neuropsychiatric symptoms present in MCI individuals, predict conversion to dementia of any cause, the significance of depression in this regard was only demonstrated by epidemiological, but not clinical studies (221). Unbalanced diet, smoking, hearing loss (142), and more recently recognized factors: excessive alcohol intake, head trauma, and air pollution (211,224) contribute to the development of AD, as well. The lifestyle interventions needed to prevent the development of AD or delay cognitive decline, stem from this knowledge and evidence of risk factors. Among all indicated, the importance of treating hypertension, lipid imbalance and obesity has been emphasized, as well as addressing hearing loss in midlife, to minimize the risk of AD development (142,216-218,221,225). It has been demonstrated that after the age of 65, encouraging physical activity, social contact and stopping cigarette consumption is of particular significance in this regard, together with the treatment of diabetes and depression (209,214,221-223,226,227). Introducing a Mediterranean type of diet could reduce the risk of conversion from an amnestic type of MCI to dementia, according to a recent meta-analysis (221,227). In addition, for an individual who has entered the AD continuum, the time it takes to progress until dementia, also largely depends on the risk factors (228). Non-carriers of APP/PSEN genetic allele mutations, who have high cognitive reserve and low-risk lifestyles are considered as having the capacity to compensate for biological changes caused by AD-P and delay the manifestation of cognitive impairment (228). In conclusion, timely addressing of modifiable risk factors is very important, since literature evidence indicates that it leads to the prevention of probably one-third of dementia cases (206,209). Preventive strategies should be applied in midlife - before

the biological evidence of AD. This means that a modern person should have a developed awareness of the incidence and possibility of developing age-related diseases, including AD. They should lead a life in accordance with the idea of preventing these diseases, already in their forties. So far, this is the best, potential solution regarding AD.

1.5. The need for answers and the need to act

There was a strong need for scientific progress in the AD field. This has been identified as one of the leading, global biomedical priorities in the past few decades. A significant breakthrough in understanding a pathophysiological concept, development of biomarkers and disease-modifying therapy is evident. However, a unified explanation, pathophysiological and molecular signature of AD, which would open the possibility for the development of causative therapy, remains a challenge. In addition, the translational concept of the knowledge achieved is still not successful.

Considering the overall pathophysiological context of AD that is known so far, it can be concluded that although many aspects are well-defined and well-argued, the multitude of these findings mainly represent pieces of the AD puzzle, that has not yet been put together. Therefore, to reach clear and unified conclusions, there is a need for progress, not only in the elucidation of new but also in the interconnection of known AD mechanisms. Consequently, that would lead to new opportunities for the application of this knowledge in clinical practice.

The current difficulty of AD identification in a timely manner lies in the facts that pathological and clinical features of the disease are in temporal discrepancy and that clinical tests provide the diagnosis only when the first symptoms appear. The development of biomarkers represents revolutionary advances in the early detection of the disease because they can detect AD neuropathological changes before clinical manifestations. They were initially introduced in clinical guidelines in 2011, but only as an optional, not necessarily diagnostic tool (138-140), since they are frequently unavailable, obtained invasively or have no clear cut-off values for determination of CSF-derived biomarkers. Revised criteria published in 2018 emphasize the significance of their clinical application and biological

confirmation of the disease (30), as described above. At that time, literature data clearly indicated high heterogeneity and complexity of AD, clinical overlaps with other neurodegenerative diseases and even with cognitive decline related to physiological aging (5,6,97-101,132,134-137). Eventually, it was estimated that with purely clinical assessment, up to 40% of AD cases may be misdiagnosed (144-150). Despite these facts, in 2018, the use of biomarkers was limited only to research (30). Although critically needed, it is clear that the present biomarkers will not be included in the diagnostic criteria, mainly due to invasiveness or the unavailability of sophisticated methods used for their detection.

Therefore, if biomarkers are not available for the population-wide routine clinical application, the question is, how can we expect to make the early diagnosis of AD? How can we be absolutely sure about the accuracy of the diagnosis, even in a symptomatic AD patient? Given that the new disease-modifying therapy may be applied only in the early AD, and identification of eligible patients further requires the availability of sophisticated imaging, another question imposes itself: Who are the new therapeutic modalities actually intended for? Currently, they are intended for the lucky minority patients who have the opportunity to enter the diagnostic procedure in specialized, large memory clinics. Also, is it realistic to expect widespread use of this therapy in the future at all? Thus, the identification of new, widely-available, simple and non-invasive biomarkers which would reflect the pathophysiological process of AD, is urgent. They would enable screening and early AD detection, and also, facilitate and popularize the application of available disease-modifying therapy. Eventually, they could also inspire research into new, causal treatments for AD.

Finally, it is important to point out, that not only is there a need to develop tools for AD diagnosis in the asymptomatic stage, but also to emphasize the problems regarding AD detection at an early symptomatic stage. Namely, literature evidence implies that the significance of cognitive problems in the wide population has not been sufficiently recognized (1,19). Thus, it seems that there is also a need to raise public awareness of the manifestations of AD and the importance of its early detection. Moreover, stigmatization of the AD population is still widespread (229). Significant global initiatives have been launched in this regard recently, but this must be a continuous task. Summarized, continuous education

and the fight against stigma in the population must not be overshadowed by large-scale research tasks, of discovering biomarkers and new treatments. Progress in these aspects will certainly increase the number of those individuals with cognitive issues, who will seek medical help and thus, raise the chance to diagnose AD in the earlier stages. Finally, these are areas where we can already act and expect benefits, while we wait for the progress in clinical management of patients with AD, through the development of new biomarkers and causal therapeutics.

1.6. What is the scientific context of this study, as a tool in the search for answers?

In the absence of a therapeutic solution and fatal outcome in each AD patient, one of the priorities is to find the tool for the early detection of the disease, so that the available, disease-modifying therapies, may be timely administered. It may also contribute to the discovery of causal treatment, extension of life expectancy and improvement of life quality of AD patients. In a multitude of research that has flooded the AD field, with the main task to identify the biomarker of the early disease stage, through the non-invasive, widely available method, there is a need for an innovative research approach.

The innovation and advantage of the present study are based on its design. It offers a new and broader research context, for the identification of the potential AD biomarker. It is based on the fact that two different diseases, AD and cancer, have been shown to have inverse incidences, as well as complex molecular relationship. Small, non-coding RNA molecules - microRNAs (miRNAs), are deeply implicated in both diseases and proposed as having the biological complexity to explain such a challenging relationship between AD and cancer.

In summary, AD, cancer and miRNAs are unified through the innovative research approach, which could potentially result in the discovery of a new biomarker for AD. This is the scientific context that will be discussed in more detail below.

1.6.1. Inverse relationship between Alzheimer's disease and cancer

It is well known that the main pathogenic mechanisms of AD and cancer are opposite in their nature: AD is characterized by apoptotic cell death and cancer by uncontrolled proliferation (29,230). Therefore, many pathways, regarding cell death, growth suppression, proliferative signaling, replicative immortality, DNA damage repair, angiogenesis, as well as some genetic factors, like tumor protein p53 (*TP53)*, have been suppressed in one but increased in the other disease (29,31,32,38,51,230,231,232,233). However, some pathophysiological processes, such as inflammation, have been widely recognized as the driving mechanism of both diseases (66,71,231,234). Nevertheless, the role of inflammation in these diseases is complex, thus, at the level of signaling pathways, some have been demonstrated to be inversely deregulated in these two diseases (235,236,237,238,239). Deregulation of cellular energetics and immune functions represent the important, common determinants of AD and cancer, but it has been demonstrated that they might be either increased or decreased in both diseases (51,77,82,231,232,240,241). This is certainly due to the complexity of these mechanisms in the pathological context of AD and cancer and might be related to the disease stage and type of cancer, too (238,242-244). However, literature data are still inconclusive in this regard.

In the last years, interestingly, the growing scientific evidence indicates that the incidences of AD and cancer are also in inverse relationship (232,245-248). Namely, epidemiological studies show that individuals with a history of cancer have a lower risk for the occurrence of AD, and vice versa. In addition, incidences of both diseases increase exponentially with age (246). A recent meta-analysis reported that the significantly reduced risk of AD has been associated with the history of cancer, without detected significance between study heterogeneity and publication bias, which goes in favor of the data relevance (248). However, in the analysis of cancer risk, a follow-up period of less than 5 years, for AD patients, has appeared as a source of heterogeneity. So, although the lower risk of cancer in AD patients was significant, the previous data reflect important issue and objective limitation for such epidemiological studies, regarding the life expectancy of AD patients, which influences the length of the follow-up period (248). It is interesting that individuals who previously had two

cancers of different origin, had older mean age at the onset of AD, compared to individuals who previously had one or no prior cancer (249). This finding increases the strength of the observed epidemiological relationship between these two diseases, also indicating a kind of "dose-dependent" effect, regarding this protection against AD. There have been attempts to identify potential differences among cancer types, in the context of the inverse incidences of AD and cancer. For example, a large Framingham study has shown that the risk of AD was lower among survivors of smoking-related cancers, compared to survivors of non-smoking related cancers (245). A negative association between AD and other cancer types has been also found for breast, oral cavity, kidney, prostate, colon, and rectum cancers (246,248-251). Reduced incidence of colorectal adenocarcinoma (CAC), which is the focus of this study, has been reported for AD patients and vice versa (235,246,252-254). The decision to include the CAC cancer group in the present study, among those showing an inverse relationship with AD, was not only guided by the fact that CAC is the third most diagnosed cancer (255), but also by the inclusion criteria and inherent course of the disease, making it more feasible for long-term studying. For example, in contrast to lung cancer, a disease often characterized with faster progression and worse prognosis, in individuals diagnosed with CAC, disease progresses slowly, prognosis is usually better, and in some early diagnosed cases, does not even require radio- and chemotherapy, which could influence gene expression. In addition, there are also findings emphasizing the significance of chemotherapy applied in CAC patients, on the reduced AD incidence (256). However, the literature data point to contradictions in this regard (257).

The precise mechanisms underlying this negative epidemiological association between AD and CAC have been investigated (258), but remain mainly unknown. Transcriptomic meta - analyses, for example, have revealed opposite regulation of a number of genes shared by AD on one, and lung, colorectal and prostate cancer on the other side (259). Also, many common pathways, responsible for the pathogenesis of both diseases, were identified as inversely dysregulated in AD and in different types of cancer (232,233,235,237,250,252,260-265). Among the most reported in this regard, are the peptidylprolyl cis/trans isomerase NIMA-interacting 1 (PIN1) enzyme (262,263) and Wnt cell survival pathway (264,265), both

overexpressed in cancer, but down-regulated in AD. It should be emphasized, that inflammation-associated genes and pathways are probably dominant, among those shared by these two diseases (234-236,238,239,266).

To summarize, the new findings about inverse incidences of AD and CAC have brought a different and challenging context to the previous knowledge about shared biological mechanisms involved in these diseases. Being mainly opposite in its nature, it has been postulated that these mechanisms might actually represent different sides of the same path, explaining opposite incidences of AD and CAC. In other words, if dysregulation of some of the common AD – CAC signaling pathways leads to a higher risk of cancer, the risk of AD will be reduced in the same individual, or vice versa.

1.6.2. MicroRNAs involved in Alzheimer's disease and colorectal adenocarcinoma, as the potential biomarkers of AD

In search of a non-invasive and easily available AD biomarker that could become widely clinically applicable, a significant number of the molecular markers in serum or plasma of AD patients has been identified, including the miRNAs (166-191,267-269).

MiRNAs are small, endogenous, highly conserved, non-coding RNAs (270,271). Their important role is to regulate the expression and translation of the protein-coding genes (270,271). It has been estimated that around 1-4% of the human genome encodes these 19-25 nucleotide long molecules (272). Their biogenesis starts with primary transcripts in the nucleus and continues through canonical or non-canonical pathways, with the final transport in the cytoplasm and cleavage by Dicer RNase enzyme, where a mature miRNA is formed (275; Figure 1). There, included in the RNA-induced silencing complex, miRNAs exert their biological role, through binding to the 3' untranslated regions (UTRs) of messenger RNA (mRNA) targets. Finally, miRNAs negatively impact gene expression through the inhibition of translation or complete degradation of the mRNAs and this is considered as the canonical mode of miRNAs action (270-276; Figure 1). Unlike known pathways of biogenesis, recently described unconventional/non-canonical mechanisms of action of miRNA molecules are still

not completely understood (275,277,278). However, it is known that their additional regulatory modes include intranuclear transcriptional regulation (279) contributing to gene silencing, but also mediating phenomenon of RNA activation (277). Finally, interaction of free miRNAs with proteins has been described, however, many explanations of how it impacts their activity are currently missing (279,280). It has been reported that each miRNA regulates the expression of more than one hundred transcripts (272), thus affecting up to 30% of the human protein-coding genes (272,273). These facts certainly reflect the complexity of miRNA roles and the possibility to impact a great number of biological functions (272,276). In fact, perceived as having the potential to shed light on many biological mechanisms, miRNAs have attracted the attention of researchers, especially in the last decade. Easy detection in both, tissues and body fluids (281), represents an additional value of these molecules and contributes to their translational potential. Moreover, miRNAs in plasma are stable molecules, which can be identified and quantified using the widely available method of the polymerase chain reaction (PCR) (281). This has also caused a big interest in studying their role as the diagnostic, prognostic biomarkers and predictors of drug response (267-269,274,282-284). The ability of miRNAs to pass the blood-brain barrier (285), was particularly advantageous for the investigation of their roles in CNS, considering not only the limitation regarding brain and spinal cord biopsy but also the fact that the lumbar puncture in order to obtain cerebrospinal fluid, is also invasive procedure.

MiRNAs have been shown to be involved in fundamental cellular processes, such as cell proliferation, differentiation, migration, and apoptosis (271,273,275,286-288). Biological pathways mediating pathological changes at the cell level, such as oxidative stress, mitochondrial dysfunction, inflammation, or telomere shortening, which accelerate cell aging, seem to be strongly regulated by miRNA molecules (269,272,275,286,288). Related to that, a series of studies have demonstrated their implication in a variety of human brain dysfunctions such as innate immunity, neuroinflammation, dysregulated amyloid metabolism and oxidative stress (285,286,288-290). In fact, these processes represent the backbone of the different AD theories (29,32-34,51,63,66,73,77,82,90), so the studying of the miRNA roles in this disease has been recognized as a promising research direction.

Actually, a number of the miRNA interactions with the key genes involved in the pathogenesis of AD have already been identified (268,290,291). Moreover, the evidence showing that some of them have been implicated in more than one pathophysiological pathway of AD does not surprise, knowing that each miRNA simultaneously acts on many different targets, often involved in different biological pathways (292,293). Eventually, as is already emphasized, there is a need to interconnect various proposed mechanisms of AD, in order to fully explain its pathophysiological cascade. These facts together, explain the inherent potential of miRNAs to emerge as the key point of AD pathogenesis. Furthermore, a number of miRNAs have been already recognized as potential AD biomarkers (267-269,290,291,294).

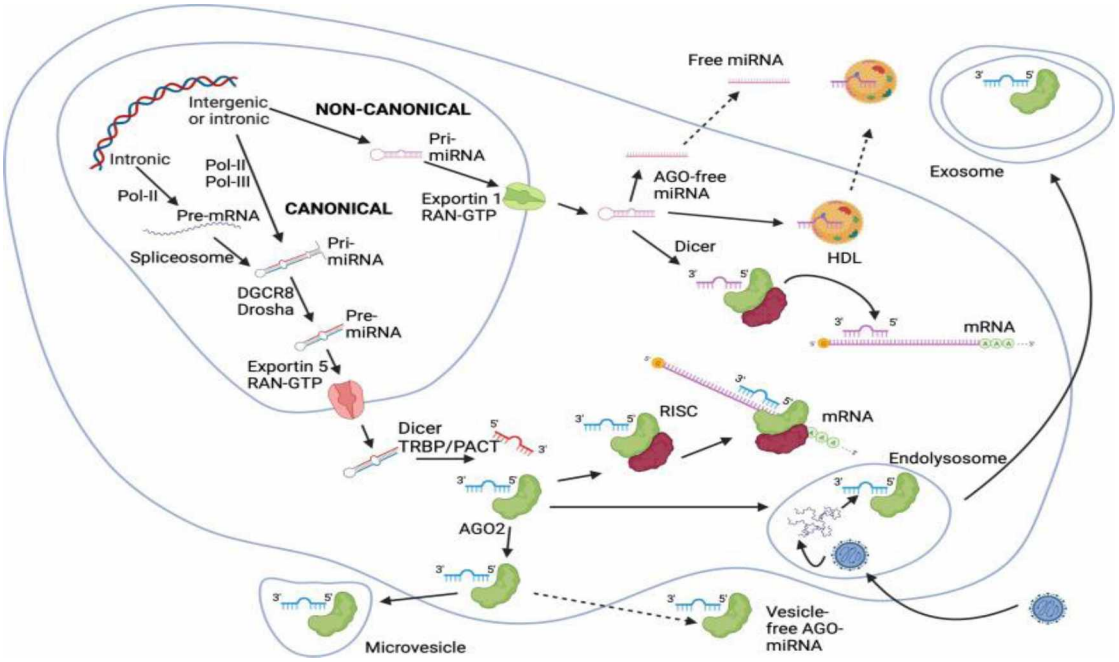


Figure 1. MiRNAs - biogenesis and modes of action. In the canonical pathway, pri- miRNAs are turned into pre-miRNAs by the action of DGCR8 and Drosha within the nucleus. Intronic miRNAs can originate from host mRNA transcripts and processed into pre-miRNA by the spliceosome. Pre-miRNAs are exported into the cytoplasm through an exportin-5/RanGTP-dependent way, and are processed into mature miRNAs by Dicer with eventually RNA binding protein cofactors TRBP or PACT. In non-canonical pathways, shRNAs are cleaved by the DGCR8/Drosha complex and exported into the cytoplasm by exportin-1 before Dicer

processing. Mature miRNAs bind to AGO proteins forming RISCs, which in turn silence or cleave mRNAs. Alternatively, miRNA-AGO complexes are exported out of the cell *via* vesicles (exosomes or microvesicles) or as vesicle-free complexes. miRNAs binding to HDLs are actively secreted. AGO-free miRNAs can be exported out of the cell as well. AGO, Argonaute; DGCR8, DiGeorge syndrome critical region gene 8; HDL, high density lipoproteins; miRNA, microRNA; mRNA, messenger RNA; PACT, protein kinase RNA activator; pre-miRNA, precursor-miRNA; Ran, Ras-related nuclear protein; RISC, RNA induced silencing complex; shRNA, small hairpin RNA; TRBP, transactivation response RNA binding protein. Source: *Antonakos N et al., 2022* (274).

The impact of miRNAs on the main aspects of the pathogenesis of both, AD and cancer, has been unambiguously shown (295). The main pathological features of cancer and AD are uncontrolled cell proliferation and apoptotic cell death, respectively (258). Being well documented as the leading regulators of cell proliferation and differentiation, migration and apoptosis, miRNAs may be the key points of the pathophysiological process of both diseases (271-273,285,286,288,289). Therefore, in order to understand whether the inverse incidence of AD and CAC is a reflection of the dysregulation of common signaling pathways, the roles of miRNAs have been investigated (295,296). The literature is rich in evidence on the impact of different miRNAs in inflammation, oxidative stress, mitochondrial dysfunction, angiogenesis, and vascular and endothelial dysfunctions in both, AD and cancer (267-269,290,291,295-298). However, these studies have mainly identified the significance of miRNAs in particular signaling pathways and molecular pathological changes, independently - either in AD, or in CAC. At the time the present research was initiated, there were very few papers investigating these roles in the context of a molecular relationship between AD and cancer.

It was a challenging task to select miRNAs for the planned investigation, considering the diversity of their simultaneous roles, as well as the broad context of the present research. In fact, this implied the need to narrow the choice of miRNAs, on those involved in a very few AD-CAC overlapping pathological cascades. It was considered that such an approach will contribute to the in-depth analysis, increase the possibility to detect important molecular

interactions between AD and CAC and define the molecular signature of this relationship through the specific expression pattern of selected miRNAs. Hopefully, that will also help to identify those molecules which have key significance in AD and the potential to become biomarkers. The most dominant theories of both diseases are based on two closely interconnected processes - innate immunity and inflammation, and thus, they have been chosen as the pathophysiological focus of the present study. Pathogenesis, as well as the progression of AD, are considered particularly determined by neuroinflammation and immune defense factors (66,71-75,78-89,92-94,108). All the miRNAs selected for the study have documented roles in immune-related and/or inflammatory pathways: miR-29a, miR-101, miR-125b, miR-146a and miR-155.

MiR-146a and miR-155 are highly involved in inflammatory biological cascades and often labeled as inflammation-related miRNAs, which reflects the dominant, among their other roles. **MiR-146a** has been among the first identified regulators of an immune system (299). At the same time, it was probably the first found to be overexpressed in the brain regions affected by AD and related to the induction of neuroinflammation (300-302). As a mediator of inflammatory signaling, dysregulation of miR-146a has been demonstrated in CAC and other cancers (303-305). The data are inconclusive regarding the understanding, of whether it is a driver of inflammatory process or a protective factor in cancers; however, further examination of its roles in CAC and AD is considered as potentially highly valuable.

Dysregulation of **miR-155,** related to physiological aging and cellular senescence, has also been shown to reflect an "inflammatory environment" related to aging (306-308), especially in the brain. Long-term inflammation has been shown to precede AD as well as CAC. The significance of its deregulation has been confirmed in AD (308-310). Inflammation-based tumorigenic role and up-regulation of this miRNA has been reported for CAC, as well (311,312). Therefore, reflecting the low-grade prolonged inflammation as increased susceptibility for the disease, investigation of miR-155 was considered as an interesting direction and to have promising potential to mark early biological changes in AD.

Apart from the significance in inflammatory cascades, the literature evidence indicates the oncosupressive role of **miR-101** in CAC and other cancers (313,314), as well as its deep

involvement in the process of amyloid accumulation (315,316), which is known to be the hallmark of AD pathogenesis (31-34). It should be noted that the molecular context of these miR-101 roles in carcinogenesis and amyloid plaque formation has been based on inflammatory cascades (313,317-319) but some other mechanisms have been proposed as well (313-316,320,321). Considering the persistent, irrefutable significance of the amyloid hypothesis, together with the recent disease-modifying therapies for AD based on it, the choice of this miRNA is considered highly relevant, regarding the aim of the present study. The promotion of inflammation and development of CAC was found to be associated with the increased expression of **miR-29a** (322). On the other hand, miR-29 up-regulation was related to neuroprotection in AD (323-325), where it has been demonstrated to mediate the process of Aβ accumulation, through the regulation of beta-site APP-cleaving enzyme (BACE) expression (323). This perceived opposite action of miR-29a in two diseases, was a good starting point for the kind of investigation proposed in this study. Interestingly, it has been also shown that the high miR-29a expression in CAC patients was in correlation with their longer disease-free survival (326), which might be an interesting aspect, considering the aim to understand the mechanism for the reduced AD risk in cancer survivors (245-248,251-254,256).

High expression of **miR-125b** found in AD patients (327) has been shown to induce inflammation and oxidative stress, promote apoptosis, and highly contribute to tau hyperphosphorylation and accumulation (328,329,330), which is the well-known pathomorphological determinant of AD (31,32,35-37). There is a high level of agreement in the literature, that its dysregulation contributes to neuronal dysfunction in AD (328-331). Although there are contradictory findings regarding miR-125 b's role in cancers (332), its implication in immune and inflammatory cascades has been unambiguously demonstrated (333). Moreover, miR - 125b has been targeted as the one that strongly reflects the interconnection between AD and CAC (295,296). Therefore, a comparison of its expression level between AD and CAC patients in this study, together with the clearer data in AD, might highlight a more specific direction of conclusions regarding CAC.

In summary, the challenge was answered by choosing those miRNAs, which, not only have a documented role in AD and CAC, but rather, each of the selected miRNAs has at least one target involved in specific pathophysiological processes, shared by both diseases. Further, considering that the miRNA roles in cancer have been investigated much longer, the principles observed, might help to elucidate their mechanisms of action in AD and potentially identify the biomarker of AD. In addition, the possibility to use these molecules as non-invasive biomarkers contributes to the particular translational value of the whole research context presented here. Finally, this is the first research of its kind in Montenegro. Literature evidence indicates that susceptibility to AD and cancer may differ among different populations (334-336), and racial and ethnic differences in the expression of miRNAs have been reported as well (337-339). So, apart from the expected universal scientific significance of this study, it should not be ignored that it may also be of special importance for the Montenegrin population.

2. THE AIM OF THE RESEARCH

The aim of this research was the identification of specific circulatory miRNAs as potential biomarkers for AD.

Using miRNA for this purpose represents an innovative approach for the stratification of the population, according to susceptibility for AD.

Hypotheses:

1. The expression level of the circulatory miRNA correlates with the level of cognitive decline in patients with AD.
2. The selected miRNAs, isolated from the plasma, could identify people suffering from AD through the specific pattern of expression.
3. The pattern of the selected miRNA expression in patients with cancer will confirm on the molecular level that there is an inverse correlation of the cell signaling pathways between AD and cancer.

3. MATERIALS AND METHODS

This research was a part of the scientific project titled: "New methods for risk stratification for progression of cancer and Alzheimer's disease in patients in Montenegro (DEMONSTRATE)". The project was coordinated by the Faculty of Medicine of the University of Montenegro and financed by the Ministry of Science of Montenegro (grant ref. 01-781).

The study protocol was approved by the Ethical Committee of the Clinical Center of Montenegro (CCM) (No. 03/01-11417/1) and by the Committee for Medical Ethics and Bioethics of the Faculty of Medicine of the University of Montenegro (No. 3824/4). All the procedures were conducted in accordance with the Declaration of Helsinki.

The research was conducted at the CCM and the Center for Scientific Research of the Faculty of Medicine, in cooperation with the Institute of Biomembranes, Bioenergetics, and Molecular Biotechnologies, National Research Council, Bari, Italy.

This study was translational and multidisciplinary. It was of a retrospective-prospective type, conducted between September 2019 and December 2021, and included:

1) Recruitment of the patients and group assignment

2) Clinical evaluation

3) Molecular biological research: quantification of miRNA expression

4) Statistical analysis of the data

3.1. Recruitment of the patients and group assignment

The present study enrolled 54 subjects, out of total 75 examined individuals (Figure 2). They were divided into four groups: 18 healthy controls (CTRL group), 9 patients whose cognitive performance was in the MCI range, according to the Montreal Cognitive Assessment – MoCA test (low-performance MoCA group - LP-MoCA), 12 patients with previously diagnosed AD (AD group) and 15 patients prospectively diagnosed with CAC (CAC group). Written informed consent to participate in the study was obtained from all participants or their legal representatives (Attachment 1).

Excluding criteria were defined before the recruitment procedure. For all participants excluding criteria were: the presence of neurological disorder (other than AD, for the AD group), psychiatric, poorly controlled chronic diseases, malignant disease (other than CAC for the CAC group), history of drug and/or alcohol abuse, and current acute illness. The geriatric depression scale - 15 (GDS-15) was performed to exclude depressive disorder. Patients who scored 9 or more points on GDS-15, suggesting major depression disorder, were excluded from the study. In addition, in healthy control subjects, neuropsychological screening test results (Mini-Mental State Examination - MMSE and MoCA) below 26 were considered as excluding criteria. Nine healthy participants who subjectively did not have a cognitive impairment, but whose MoCA score was lower than 26, formed MCI group, as previously stated. Therefore, in these participants, cognitive impairment was accidentally discovered. Exclusion criteria for the CAC patients were preoperative adjuvant therapy, clinically diagnosed hereditary adenomatous polyposis or hereditary non-polyposis CAC, as well as a history of malignant disease, as these factors could potentially introduce bias and influence the objectivity of the results. CAC patients with cognitive decline on neuropsychological screening tests were also excluded from the study. The recruitment process in accordance with the mentioned criteria is presented in Figure 2.

Participants from the control and LP-MoCA groups were volunteers, recruited at the Faculty of Medicine. AD patients were recruited during their regular follow-up appointments, at the

Neurology Clinic of the CCM. According to the detailed evaluation, including repeated neuropsychological and neurological assessments at regular follow-ups, the AD group involved patients at different cognitive stages - from those at the level of MCI to severe dementia. Therefore, in order to detect the potential significance of the investigated miRNAs at the earliest disease stage, the AD group was divided into 2 subgroups during the recruitment process: patients in the early symptomatic disease (EAD), which includes MCI due to AD as well as mild dementia stage - MoCA score ≥ 17 and advanced AD (AAD), with MoCA score < 17, which includes moderate and severe dementia cases (68,138-140). Recruitment of CAC patients was carried out at the Center for Digestive Surgery at the CCM in the preoperative period for surgical resection of the colon tumor previously diagnosed by colonoscopy. Pathohistological analysis of the samples taken during the surgery was carried out and confirmed a final diagnosis of the disease.

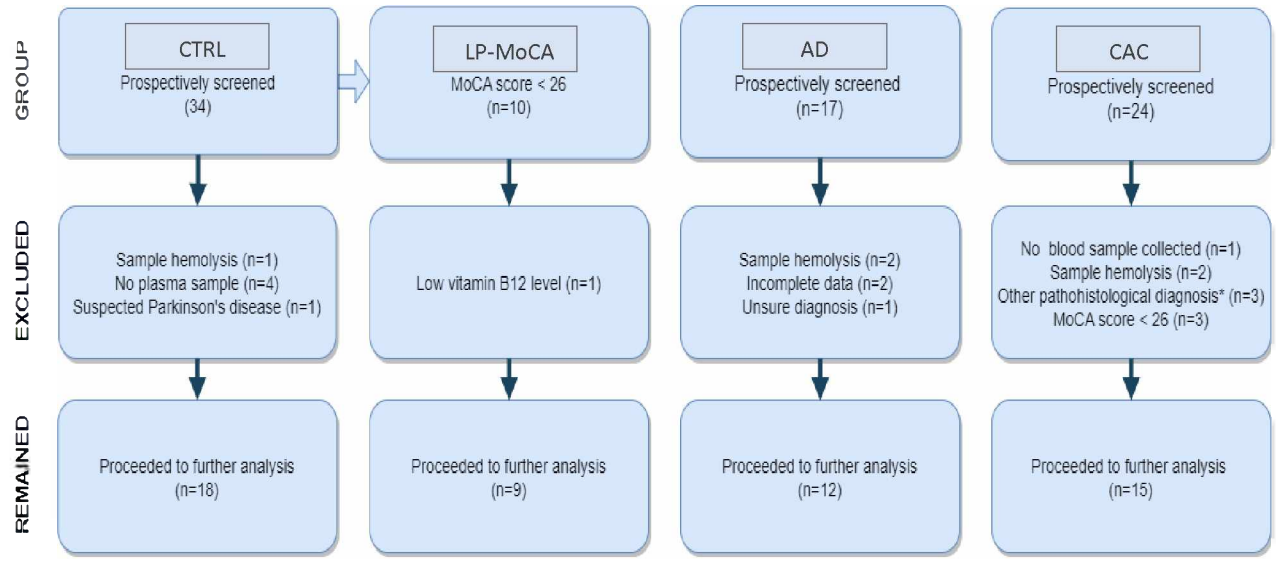


Figure 2. Recruitment process. CTRL = healthy volunteers with normal cognitive function; LP-MoCA = healthy volunteers with subjectively normal cognitive function, but whose cognitive performance was lower - in the MCI range, according to MoCA test; AD = participants diagnosed with AD; CAC = examinees diagnosed with colorectal adenocarcinoma, *Adenoma (n = 2), Ulcerative colitis (n = 1).

3.2. Clinical evaluation

3.2.1. Clinical evaluation flowchart

In order to standardize the data collection process during the clinical interview and to obtain comparable demographic and clinical data, all the participants filled out a questionnaire designed specifically for this purpose (Attachment 2). Subsequently, appropriate physical and specialized neurological examination, neuropsychological assessment, and peripheral blood sampling were performed as outlined in Table 2. These steps were necessary to confirm the diagnosis for inclusion but also to confirm the absence of exclusion criteria for the study group.

Table 2. Summary of methods used in the clinical part of the research.

TEST	GROUP OF THE EXAMINEES
Standardized questionnaire for medical history	AD, CAC, CTRL, LP-MoCA
Physical examination	AD, CAC, CTRL, LP-MoCA
Neurological examination	AD, CTRL, LP-MoCA
Neuropsychological screening of cognitive status and depression	AD, CAC, CTRL, LP-MoCA

Peripheral blood sampling for biochemical laboratory analyses	AD, CAC, CTRL, LP-MoCA

3.2.2. Physical examination

The purpose of the physical examination of study subjects was to help in the assessment of potential exclusion criteria or to detect other diseases. This valuable diagnostic tool included: inspection of the whole body, rapid assessment of vision and hearing, palpation of lymph nodes and thyroid examination, heart and lung examinations, abdominal palpation, succussion of renal lodges, and examination of the extremities. Besides, being done after the carefully obtained medical history by using a questionnaire designed for the study, the doctor was able to perform targeted or complaint-driven physical examination, and also to request additional laboratory analyses if needed.

For patients diagnosed with CAC, a physical examination was conducted by a surgeon at the Clinic for Digestive surgery of CCM, where the patients were hospitalized for operative treatment.

For AD patients and control subjects, for whom a neurological examination was mandatory, a general physical examination was performed by a neurologist, at the Clinic for Neurology of CCM or at the Faculty of Medicine.

3.2.3. Neurological examination

An interview according to the defined questionnaire, together with a neurological examination, was conducted by a neurologist, for AD patients and control subjects, at the CCM or at the Faculty of Medicine.

Patients with AD were previously diagnosed at CCM, according to the NIA-AA criteria for routine clinical practice (138-140). At the time of the recruitment, they were neurologically re-evaluated for the purpose of research and identification of potential comorbidities defined as exclusion criteria.

All the volunteers for the study, including those who denied neurological disorders during an interview, underwent detailed neurological examination, in order to thoroughly select participants for the study and identify exclusion criteria (Figure 3). Participants who did not report SCD were not qualified for further clinical cognitive evaluation, but, according to the study protocol, were still referred for neuropsychological screening testing.

3.2.4. Neuropsychological assessment

The neuropsychological examination of all the participants was conducted by a doctor or a psychologist certified for neuropsychological assessment. It included: a short assessment of SCD, MMSE, MoCA and GDS-15 tests. In AD patients, depression was previously excluded as a differential diagnosis, so the GDS-15 was not performed again.

The present study recruited people who felt mentally and physically healthy for the control group and was not focused on the structured evaluation of SCD (155). However, subjective cognitive status was briefly assessed by open-ended questions, which is officially supported as an alternative to structured SCD evaluation (155). There were two mandatory questions: 1. “Do you have any difficulties in remembering (things)?” ; 2. “Have you experienced any changes in memory?” , usually followed by appropriate subqueries. The purpose of asking these questions was to estimate the presence of potentially neglected cases of cognitive impairment and select participants for potential further clinical evaluation of cognitive status

MMSE and MoCA tests represent valuable, objective neuropsychological screening tools, that allow the examiner to assess the degree of cognitive impairment. Both of the tests examine multiple cognitive domains: visuospatial, executive functions, multiple aspects of attention and language, abstract thinking, memory, and orientation. However, among subjects with MCI degree of cognitive impairment according to neuropsychological screening, the MoCA test score was used for their final selection, since literature data consistently confirm its potential for improved detection of MCI and superiority compared to MMSE (340). The official form of tests used can be found in Attachment 3.

GDS is a brief, depression case–finding instrument, which is proven to be sensitive and specific for detecting depression in the general population and especially useful when applied to the relatively cognitively intact population of the elderly (341) (Attachment 4). In comparison to other, even more sensitive scales used in everyday neuropsychological and psychiatric practice, like the Beck Depression Inventory, the setting and length of GDS were found to be more appropriate for the purpose of the research, which involved volunteers feeling mentally healthy.

3.2.5. Peripheral blood sampling and laboratory examination

Biochemical laboratory analyses helped to identify conditions that were among excluding criteria (Figure 2) or to check parameters of special interest for particular chronic diseases of our study participants, which would exclude them from the study (e.g. HbA1C >10). Also, the results of the laboratory tests were used to confirm the presence of some of the participants' diseases, self-reported by the participant during the recruitment interview (Table 4).

The following analyses were conducted for each study subject: complete blood cell count, glycemia, lipid status, liver enzymes, urea and creatinine, electrolyte status, thyroid function, folate, vitamin B12, and C – reactive protein as an inflammatory marker.

Until the moment of the blood sampling, the thorough selection of the examinees had been done, so that they eventually could have been included in the molecular biological part of the research.

3.3. Molecular biological research: quantification of miRNA expression

Extraction of circulatory miRNA and analysis of its expression profile performed for the purpose of this study, were conducted for the first time in Montenegro.

3.3.1. Sample processing and miRNA extraction

Ten milliliters of peripheral venous blood were collected from each participant into BD Vacutainer® Venous Blood Collection Tubes (cat. No. 367525) containing EDTA. The tubes were kept on ice and processed within 1 hour of the blood collection. Care was taken to minimize the effect of all pre-analytical variables. Plasma was separated from the whole blood by centrifugation at 1.900 × g for 10 min at 4 °C, followed by an additional centrifugation step at 3.000 × g for 15 min at 4 °C, to remove the remaining cellular nucleic acids attached to cell debris. All samples were aliquoted in RNAse/DNAse - free tubes and stored immediately at - 80 °C until further analysis. MiRNA was isolated from plasma by using miRNeasy Serum/Plasma Advanced Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, cell-free total RNA, primarily miRNA, was extracted from 200 ml of plasma by guanidine-based lysis of the sample, removal of protein inhibitors and RNases, and silica-membrane-based purification. MiRNA is eluted in 20 ml of RNase - free water. The miRNA concentration was determined using Qubit microRNA Assay Kit (Q32880, Invitrogen, Thermo Fisher Scientific) on a Qubit 3.0 fluorimeter (Q33216, Invitrogen, Thermo Fisher Scientific, USA).

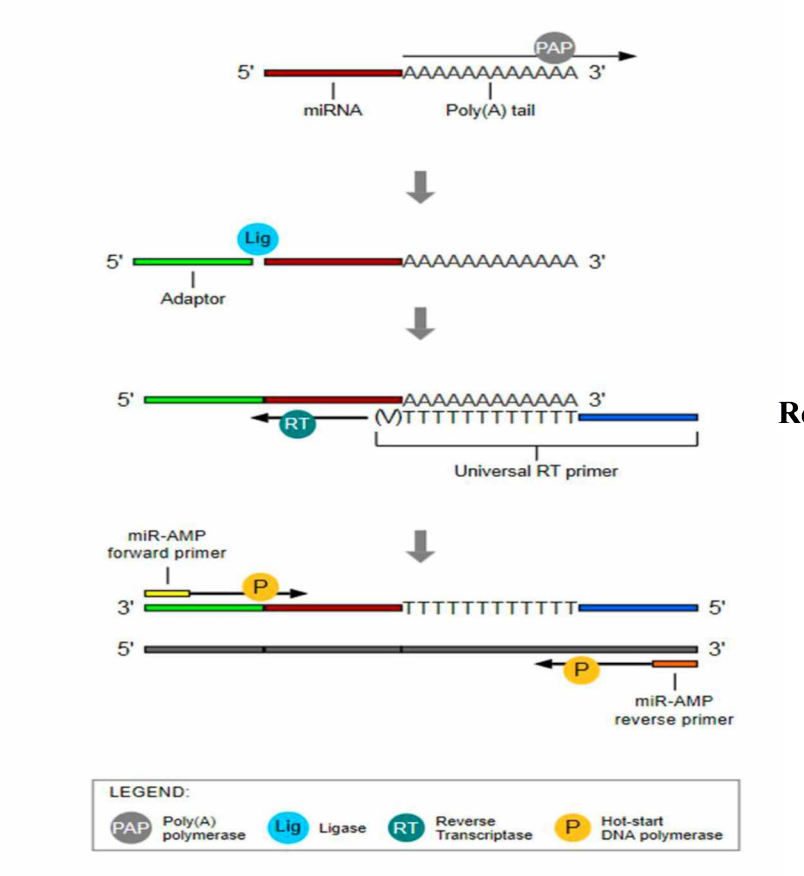
3.3.2. Quantification of miRNAs by RT - PCR

Two milliliters miRNA from each sample was reversely transcribed to cDNA using TaqMan Advanced miRNA cDNA Synthesis kit (A28007, Applied Biosystems, USA) and analyzed with TaqMan Advanced microRNA Assays (A25576, Applied Biosystems, USA) for miR - 29a/b, miR - 101, miR - 125b, miR - 146a and miR - 155. In the first step, plasma - purified miRNA was modified by poly (A) - addition to the 3' end of the mature transcript, followed by lengthening the 5' end by adapter ligation. This way modified miRNA then underwent reverse transcription, and cDNA amplification (miR - Amp reaction) (Figure 3.), followed by quantification of miRNA expression with aforementioned TaqMan Assays. qRT - PCR was run on an Applied Biosystems 7300 Real Time PCR system (Applied Biosystems, USA) with the following conditions:

Table 3. qRT - PCR – conditions

Step	Temperature	Time	Cycles
Enzyme activation	95°C	20 seconds	1
Denature	95°C	3 seconds	40
Anneal/Extend	60°C	30 seconds	40

The expression levels of target genes were normalized by using the mean expression levels of the miR - 361 - 5p gene, selected as the most stable internal control miRNA (among miR – 186 - 5p, miR - 1255a and miR – 361 - 5p) by the NormFinder algorithm (342). The expression of every target gene was calculated using the $2^{-\Delta\Delta Ct}$ method. Every sample was retrotranscribed twice and run in triplicate each time.



Poly(A) - tailing reaction

Adaptor ligation reaction

Reverse transcription (RT) reaction

miR - Amp reaction

Figure 3. Process of miRNA reverse transcription.

Adapted from:TaqMan® Advanced miRNA Assays
USER GUIDE
Single-tube assays for use with:
TaqMan® Advanced miRNA cDNA Synthesis Kit
Catalog Number A25576, Publication Number 100027897, Revision C

3.4. Statistical analysis

All statistical analyses were performed using GraphPad Prism 9.3.1. (GraphPad Software, San Diego, CA, USA) and the statistical software R. The results were considered statistically significant when $p < 0.05$. Continuous variables were first tested for normality of distribution by D'Agostino-Pearson and Shapiro-Wilk tests and analyzed with the t-test or one-way ANOVA, whereas categorical variables were analyzed with the χ^2 test or Fisher's exact test. Associations between miRNA expression and clinical variables were explored using Mann-Whitney and Kruskal-Wallis tests, as appropriate.

Pearson correlation coefficients were computed to quantify the degree to which two variables are related or to find out how much one variable tends to change when the other one does. Linear regression models were used to test whether a measurable variable was influenced by other variables. For the assessment of a single variable independently, a simple linear regression model was performed.

The statistical test of special interest for the purpose of this study was receiver operating characteristic (ROC) curve analysis, which allows for a more precise insight into the sensitivity and specificity of selected miRNAs in the discrimination of healthy and diseased individuals and assesses their potential to serve as a diagnostic test and potential prognostic biomarkers. Sensitivity refers to the fraction of people with the disease that the test correctly identifies as positive and specificity refers to those who do not have the disease and are correctly identified with a negative test. A ROC curve helps to visualize and understand the tradeoff between high sensitivity and high specificity when discriminating between clinically normal and clinically abnormal laboratory values. It actually quantifies the overall ability of the test to discriminate between those individuals with the disease and those without the disease.

4. RESULTS:

4.1. Demographic, clinical characteristics and expression level of miRNAs in the examinees of different cognitive status

4.1.1. Demographic and clinical features of the examinees

A summary of the demographic and clinical characteristics of the study participants is given in Table 4. There was no significant difference in age among CTRL, LP-MoCA and AD groups. Male and female examinees were almost equally represented in the groups. Participants of the examined groups had on average similar levels of education.

As expected, MoCA scores among the study participants were significantly different, with lower values in AD and LP-MoCA groups, compared to subjects in the control group ($p < 0.0001$, Figure 4).

Hypertension, hyperlipidemia and diabetes mellitus were the most common diseases among the study participants, but their prevalence was not significantly different among the groups. Also, the average value of BMI did not significantly differ among examinees of each group. The frequency of habits, like smoking, coffee consumption, regular physical activity and hobbies related to music, was similar among the groups.

Table 4. Demographic and clinical features of the examinees

Variables	CTRL (n=18)	LP-MoCA (n=9)	AD (n=12)	*p*-value
Age (mean ± SD)	65.44 ± 8.12	70.33 ±8.46	70.92 ±7.34	0.139
Median (range)	65.0 (55.0 – 77.0)	71.0 (55.0 – 82.0)	70.0 (59.0– 85.0)	
Gender				0.679
Male	11 (61.1%)	4 (44.4%)	6 (50%)	
Female	7 (38.9%)	5 (55.6%)	6 (50%)	
Years of education (mean ± SD)	13.72 ± 2.52	11.44 ± 3.97	11.25 ±3.05	0.079
MoCA score (mean ± SD)	27.67 ± 1.19[a]	21.67 ± 3.87[b]	15.31 ± 7.9[b]	**<0.0001*** **
Body mass index (BMI)	27.21 ± 3.88	24.23 ± 3.15	25.88 ±2.26	0.176
Hypertension	8 (44.5%)	5 (55.5%)	8 (66.7%)	0.486
Hyperlipidemia	7 (38.9%)	1 (11.1%)	5 (41.7%)	0.269
Diabetes mellitus	3 (16.7%)	1 (11.1%)	5 (41.7%)	0.176
Physical activity	12 (66.7%)	4 (44.5%)	7 (58.3%)	0.541
History of smoking	8 (44.5%)	6 (66.7%)	4 (33.3%)	0.310
Coffee consumption	12 (66.7%)	5 (55.6%)	3 (25%)	0.078
Played music	3 (16.7%)	1 (11.1%)	2 (16.7%)	0.921

SD - standard deviation; Physical activity = walking ≥ 30 min at least 5 days per week; History of smoking = current or former smokers; Coffee consumption = consumption of 3 or more cups daily; played music = practicing of any kind of music (playing an instrument, singing, dancing), currently or previously in life; * Statistically significant difference.

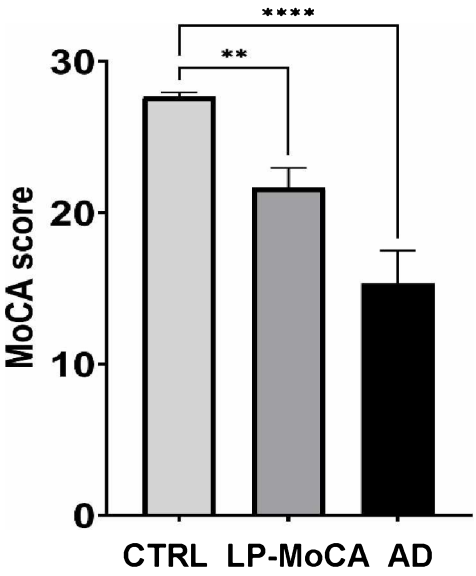


Figure 4. Comparison of the MoCA score between the study groups. CTRL, control group; LP-MoCA, participants whose performance on MoCA test was lower – in mild cognitive impairment range; AD, patients with Alzheimer's disease. * $p < 0.05$, ** $p < 0.01$.

In addition, as a part of clinical evaluation, patients filled out a standardized questionnaire that included questions particularly focused on the AD population, which explored the wider context of the diagnostic process (Attachment 1). The average time from the first disease manifestation until the diagnosis was 13.4 months and this was found to be in strong correlation with the educational status of patients. Unexpectedly, the higher the level of the patient's education, the longer it took to diagnose AD ($r = 0.6060$, $p = 0.036$; Figure 5). There was no correlation between the age of AD patients and the time to diagnosis (data not shown).

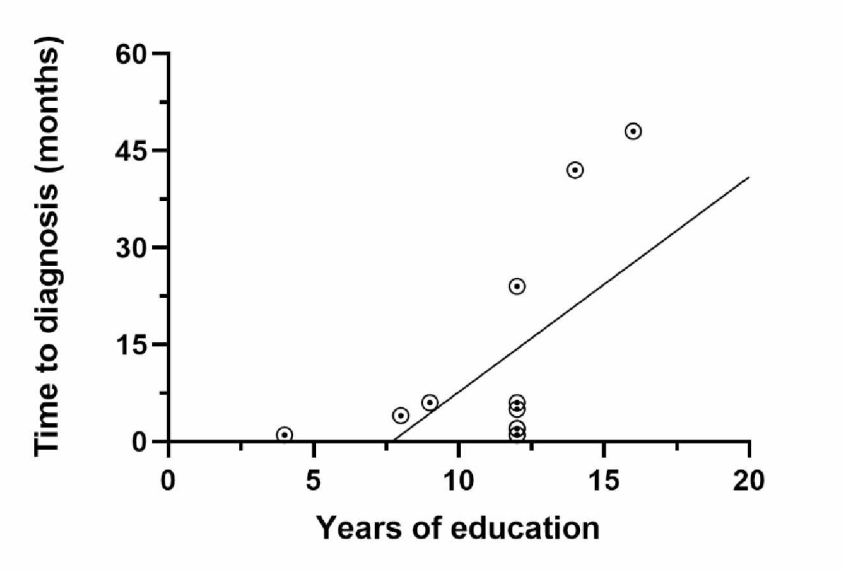


Figure 5. Relationship between years of education and time to diagnosis of AD patients.

4.1.2. In subjects without subjective cognitive decline, the neuropsychological screening score was in the mild cognitive impairment range

None of the healthy volunteers in the study reported SCD (Table 5). The percentages of the volunteers with normal cognitive performance and those who scored under 26 on neuropsychological screening tests are given in Table 5. MoCA and MMSE results were in correlation ($r = 0.725$; $p < 0.01$), but MoCA proved to be more sensitive since CI would not be discovered in 22.2 % of examinees if they were evaluated by MMSE only (Figure 6). When compared, LP-MoCA and control groups were not significantly different regarding the prevalence of depression (GDS scores > 9, $p = 0.367$, data not shown).

Table 5. Cognitive performance of the healthy volunteers

Evaluated category	Percentage of the examinees
Subjective cognitive decline (SCD)	0%
MMSE score	
26-30	88.9%
<26	11.1%
MoCA score	
26-30	66.7%
<26	33.3%

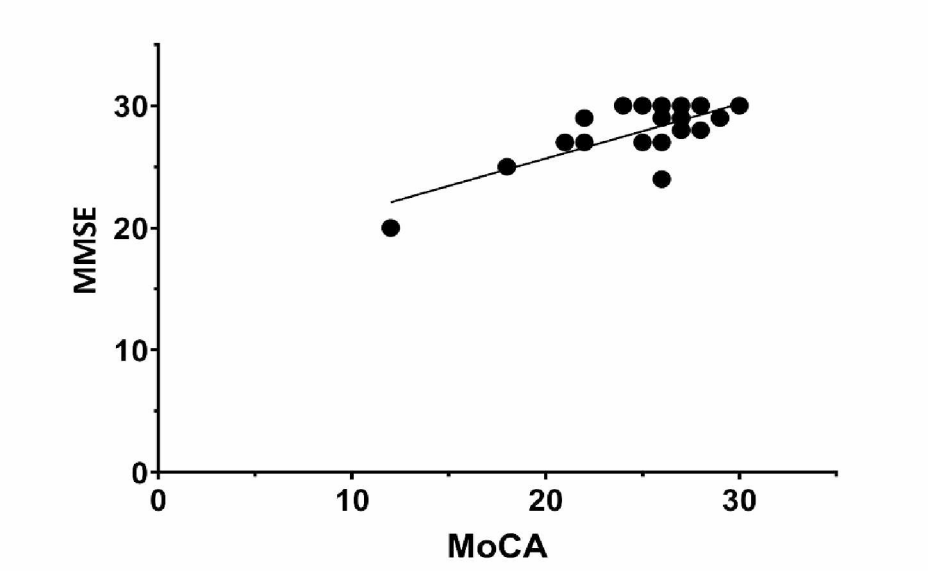


Figure 6: Correlation of Montreal Cognitive Assessment (MoCA) and Mini-Mental State Examination (MMSE) scores of the healthy volunteers.

Interestingly, our results showed that the number of years of education of the healthy volunteers was in a positive correlation with the MoCA score ($r = 0.491$, $p < 0.05$; Figure 7).

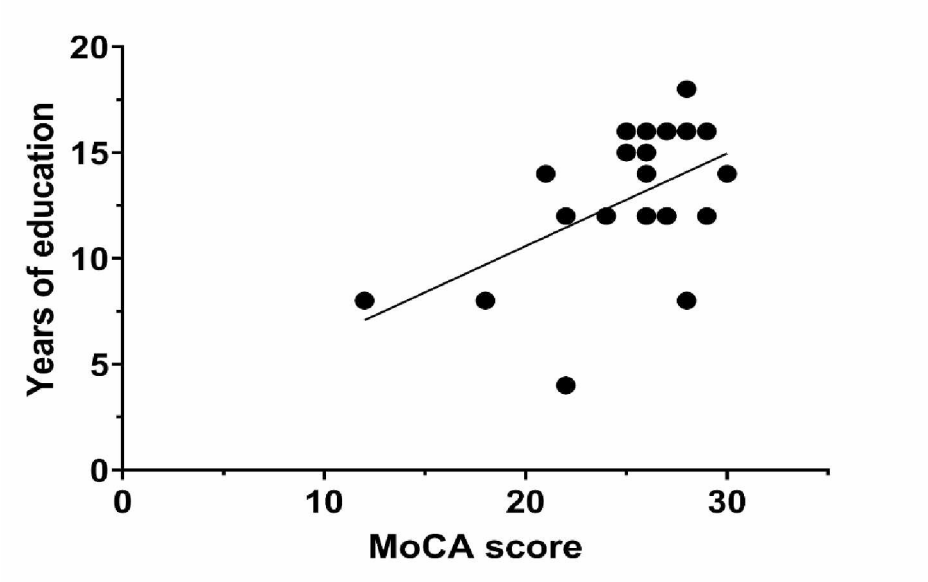


Figure 7: Correlation of education and MoCA score of the healthy volunteers.

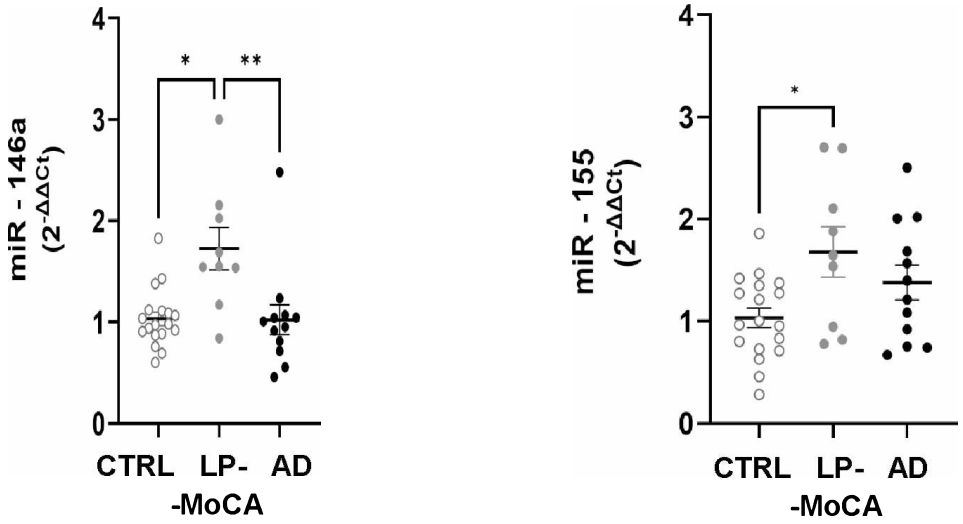
Neurological examination of the control and LP-MoCA groups did not indicate the presence of central nervous system disease. The participants denied a history of cerebrovascular or other neurological disease that could cause cognitive decline in the LP-MoCA group. Also, biochemical analyses of blood samples showed that none of the volunteers included in the study had thyroid dysfunction, vitamin B12 deficiency, severe anemia, or acute or poorly controlled chronic conditions that could explain this apparent cognitive decline.

4.1.3. miR-146a and miR-155 are up-regulated in subjects of the LP-MoCA group

Next, using the qRT-PCR, the expression level of the following circulatory miRNAs was determined: miR-29a, miR-101, miR-125b, miR-146a and miR-155 in the CTRL, LP-MoCA and AD groups. All 5 miRNAs are known to be deeply involved in the pathogenesis of both diseases. Statistical analysis did not show any significant difference in the expression level of miR-29a, miR-101 and miR-125b among the examined groups ($p = 0.151$, $p = 0.437$, $p = 0.302$ respectively, data not shown).

Circulatory miRNA-146a expression levels were found to be up-regulated in the LP-MoCA group, compared to both, the CTRL ($p = 0.012$) and AD group ($p = 0.009$). The expression level of miR-146a in the control subjects, however, was not significantly different from those with AD ($p > 0.999$) (Figure 8A).

A similar pattern of expression among the groups was found for miR-155. Its expression level was significantly higher in LP-MoCA participants, compared to the CTRL ($p = 0.019$) (Figure 8B), but there was no difference in miR-155 levels between control and AD groups ($p = 0.224$).

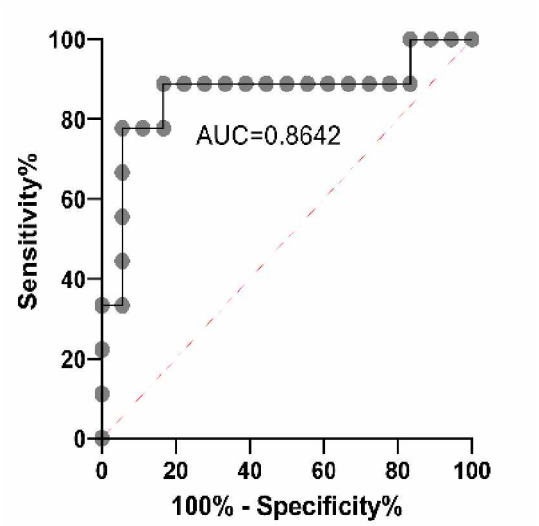


A. **B.**

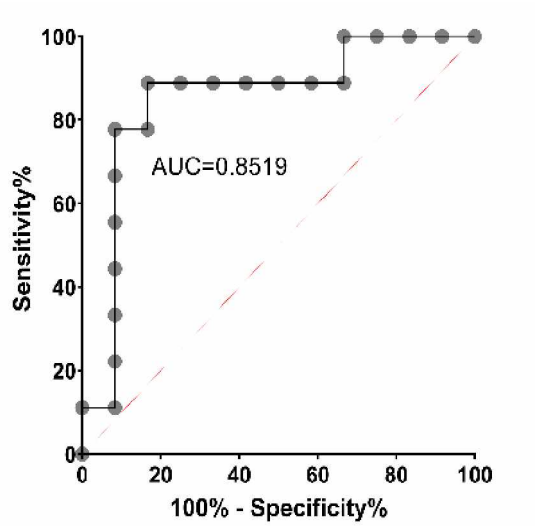
Figure 8: A. Comparison of the circulatory miR-146a expression levels **B**. Comparison of the circulatory miR-155 expression levels. CTRL, control group; LP-MoCA, participants whose performance on MoCA test was lower - in mild cognitive impairment range; AD, patients with Alzheimer's disease. * $p < 0.05$, ** $p < 0.01$.

In order to have more precise insight into the sensitivity and specificity of these miRNAs in the discrimination of healthy and those with cognitive problems and assess these miRNAs' diagnostic potential, ROC curve analysis was performed (Figure 9A - C). For the miR-146a

expression values of control and LP-MoCA groups, AUC was 0.864 (95% CI, 0.685 – 1.0), with 77.8% sensitivity and 94.4% specificity for the cut-off value of > 1.485 (Figure 9A), whereas for the data on miR-146a expression in participants with LP-MoCA and AD, AUC was 0.852 (95% CI, 0.668 - 1.000) with 88.89% sensitivity and 83.33% specificity for the cut-off value of > 1.121 (Figure 9B). When miR-155 expression level in the LP-MoCA and control groups was analyzed, AUC was 0.765 (95% CI, 0.547 to 0.983), with 66.7% sensitivity and 88.9% specificity for the cut-off value of > 1.444 (Figure 9C). Therefore, ROC curve analyses showed that both, miR-146a and miR-155, had significant diagnostic values (AUC > 0.75) and could differentiate the LP-MoCA group from healthy individuals in the control group, and miR-146a could differentiate LP-MoCA from AD patients as well.



A.



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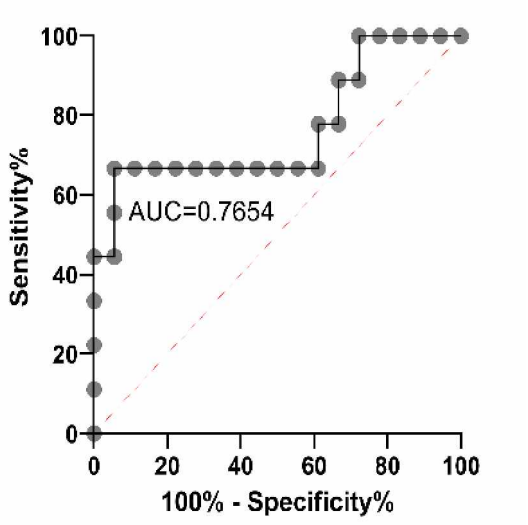


C.

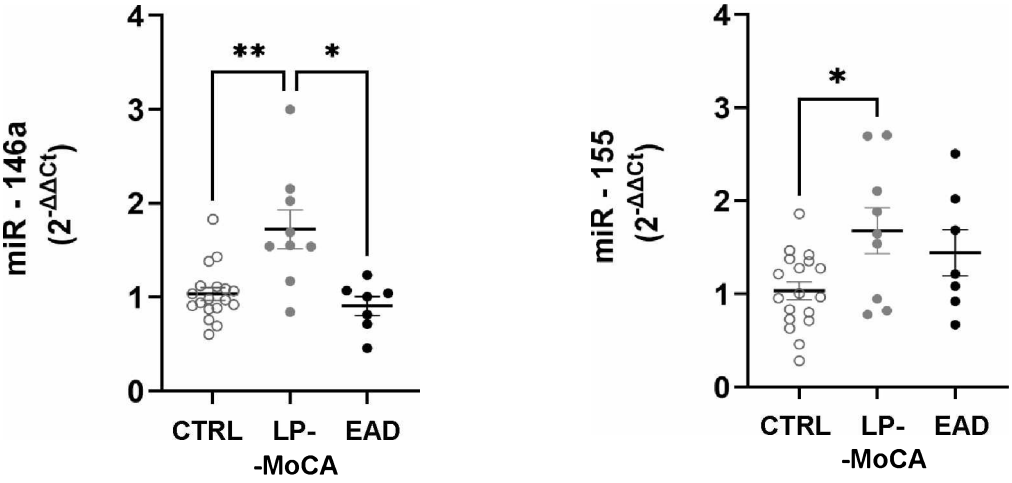
Figure 9. Receiver operating characteristic (ROC) curve analysis of altered miRNAs. **A.** ROC for miR-146a in control and LP-MoCA groups; **B.** ROC for miR-146a in LP-MoCA and AD groups; **C**. ROC for miR-155 in control and LP-MoCA groups. CTRL, control group; LP-MoCA, participants whose performance on MoCA test was lower - in mild cognitive impairment range; AD, patients with Alzheimer's disease.

4.1.4. miR-146a and miR-155 expression levels are unchanged between early symptomatic and advanced stages of AD

MiR-146a and miR-155 demonstrated the potential for detection of early cognitive impairment in this study (Figures 8 and 9). Next, we wanted to test whether these miRNAs have equal potential with respect to the early symptomatic AD phase only, given that in the present research, the AD group involved patients in the early, as well as those in advanced AD symptomatic stage. To that aim, expression values of miR-146a and miR-155 were compared among CTRL, LP-MoCA and EAD subgroups of AD patients (Figure 10), as well as between those in the early and advanced stage, within the AD group. Results demonstrated the same expression patterns for both, miR-146a and miR-155 as in respect to the whole heterogeneous AD group (p = 0.003, p = 0.022 respectively, Figure 10). The difference in

the expression level of miR-146a and miR-155 between EAD and AAD was not statistically significant ($p = 0.367$, $p = 0.688$, respectively, data not shown).

It is important to note that EAD and AAD subgroups were homogeneous regarding demographic and clinical variables (data not shown).



A. **B**.

Figure 10. A. Comparison of the circulatory miR-146a expression levels **B**. Comparison of the circulatory miR - 155 expression levels. CTRL, control group; LP-MoCA, participants whose cognitive performance on MoCA test was lower - in mild cognitive impairment range; EAD, patients in the early symptomatic Alzheimer's disease. * $p < 0.05$, ** $p < 0.01$.

Moreover, although the expression of miR-155 was not different between LP-MoCA and AD groups, the expression levels of miR-146a and miR-155 plotted together on a two-dimensional scatter plot illustrate a unique expression pattern of these selected inflamma-miRs, that differentiates LP-MoCA group from healthy individuals and from patients with AD (Figure 11).

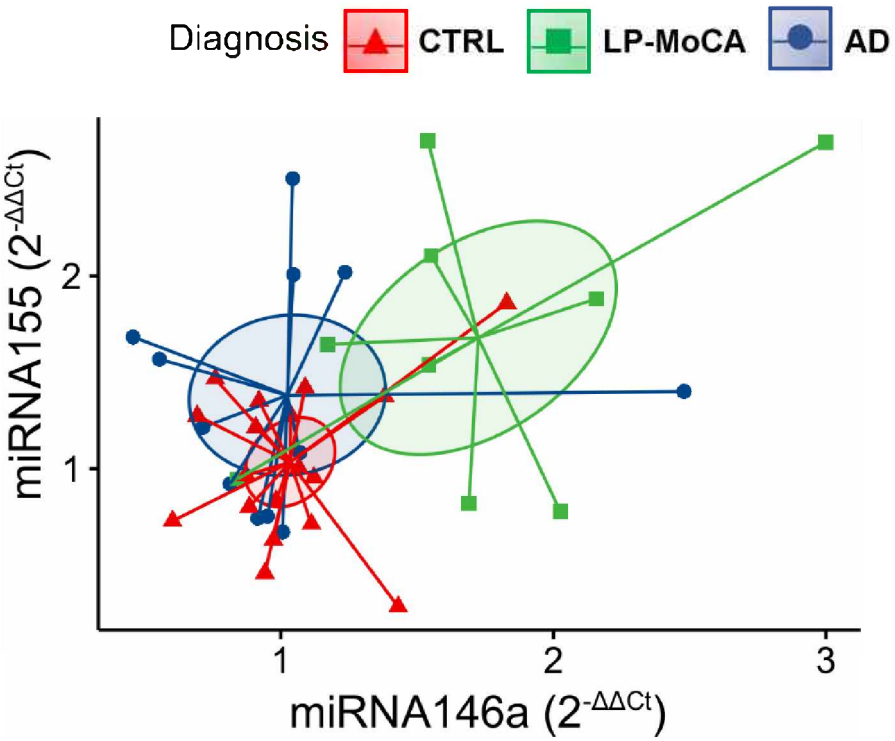


Figure 11. The joint expression pattern of the selected inflammatory miRNAs differentiates the LP-MoCA group from the other two groups of participants. The graph represents a two-dimensional scatter plot of miRNA-146a and -155 expression levels. Each data point shows expression levels of miRNA-146a and -155 in the plasma sample of one study participant, and it is labeled according to the associated diagnosis (CTRL - control, LP-MoCA - patients whose cognitive performance on MoCA test was lower – in mild cognitive impairment range, AD - Alzheimer's disease). The data clustering is illustrated by the superimposed star plot showing the mean value for a group in the center of each cluster, which is surrounded by the 95% confidence area in the shape of the ellipse.

4.1.5. Potential impact of demographic and clinical variables on the miRNA expression level

In order to further examine the potential additional impact of demographic characteristics and comorbidities of the participants on the regulation of miRs-146a and -155, a multiple linear regression statistical model was used. In this statistical model, the influence of the following covariates was examined: age, gender, years of education, BMI, hypertension, hyperlipidemia, diabetes mellitus, physical activity, history of smoking, coffee consumption, and playing music. The results showed that there was no correlation between any of these variables and expression levels of miR-146a, or miR-155 (data not shown).

4.2. The analysis of miR-146a and miR-155 target genes

In order to identify the target genes of miR-146a and miR-155, a detailed analysis of the information available in the Search Tool for the Retrieval of Interacting Genes database (STRING) was performed. Only the genes determined by the Reporter gene assay, Western blot or qRT-PCR analysis, that is, supported by the strong evidence, were included in the analysis (Attachments 6 and 7). The available data were considered in the context of the present research, therefore, the relationship with Alzheimer's disease, the immune and inflammatory response of the identified target genes, was analyzed (Tables 6 and 7). Finally, to investigate the overlapping modes of miR-146a and miR-155 action, their common target genes have been identified as well (Table 8).

Table 6. Selected target genes of miR - 146a

CASP7 [a] - Caspase - 7
NOS1 [a] - Nitric oxide synthase 1
RTN4 [a] - Reticulon 4
IL6 [a, b] - Interleukin – 6
NFKB1 [a, b] - Nuclear factor NF-kappa-B p105 subunit
PTGS2 [a, b] - Prostaglandin endoperoxide–synthase 2
TNF [a, b] - Tumor-necrosis factor
FADD [a, b, c] - FAS-associated via death domain

[a] genes involved in the pathogenesis of Alzheimer's disease; [b] AD-related genes with the role in immune and inflammatory response; [c] common target genes of miR-146a and miR-155; The indicated groups are additionally denoted with the different shades of gray.

Table 7. Selected target genes of miR - 155

BDNF [a] - Brain-derived neurotrophic factor
PALD1 [a] - Phosphatase domain containing, paladin 1
PICALM [a] - Phosphatidylinositol-binding clathrin assembly protein
SLC33A1 [a] - MFS transporter, pat family, solute carrier family 33 (Acetyl-CoA transporter), member 1
YWHAZ [a] - 14-3-3 protein zeta/delta
CD4 [a, b] - T-cell surface glycoprotein CD4
CSF1R [a, b] - Macrophage colony-stimulating factor 1 receptor
IL1B [a, b] - Interleukin 1B
FADD [a, b, c] - FAS associated via death domain

[a] genes with the documented role in AD; [b] AD-related genes involved in immune and inflammatory response; [c] common genes of miR-146a and miR-155; The indicated groups are additionally denoted with different shades of gray.

Table 8. Common target genes of miR-146a and miR-155

CFH - Complement factor H
CRP - C-reactive protein
EGFR - Epidermal growth factor receptor
FADD [a, b] - FAS-associated via death domain
ICAM1 - Intercellular adhesion molecule 1
IL8 - Interleukin - 8
IRAK2 - Interleukin - 1 receptor-associated kinase 2
MYD88 - Myeloid differentiation primary response protein MyD88
PTEN - Phosphatase and tensin homolog
PTGES2 - Prostaglandin E synthase 2
RAC1 - RAS-related C3 botulinum toxin substrate
SMAD4 - Mothers against decapentaplegic homolog 4

[a] genes involved in the pathogenesis of Alzheimer's disease; [b] AD-related genes with the role in immune and inflammatory response; Common miR-146a and miR-155 genes involved in AD and in the immune and inflammatory response are denoted with gray color.

4.3. Demographic, clinical characteristics and expression level of miRNAs among the healthy examinees, patients with colorectal adenocarcinoma and those with Alzheimer's disease

4.3.1. Demographic and clinical features of the examinees

A summary of the demographic and clinical characteristics of the participants of CTRL, AD and CAC groups is given in Table 9. There was no significant difference in age and gender structure among examined groups. Also, the groups did not significantly differ regarding the number of years of education.

As expected, MoCA scores among the groups were significantly different, with lower values in patients with AD compared to subjects in the control and CAC groups ($p < 0.0001$, Figure 12).

Participants of each group had on average similar BMI, which falls within the overweight range. Hypertension was the dominant comorbidity, without a significant difference in its prevalence among the groups. Diabetes was the second most frequent comorbidity without significantly different representation in CTRL, AD and CAC groups. None of the patients with CAC included in this study had hyperlipidemia, thus, this disorder was significantly more present in both, the control and the AD group, compared to CAC (Figure 13). Interestingly, regular physical activity was significantly higher in CAC patients compared to AD patients, but also compared to the healthy individuals ($p = 0.023$, Figure 14); coffee consumption was significantly lower in the CAC group compared to the other two groups ($p < 0.01$, Figure 15). It is important to note that study groups were not different regarding the history of smoking.

Table 9. Demographic and clinical features of the CTRL, AD and CAC groups

Variables	CTRL (n=18)	AD (n=12)	CAC (n=15)	*p*-value
Age (mean ± SD)	65.44 ± 8.12	70.92 ± 7.34	64.07± 7.43	0.079
Median (range)	65.0 (55.0 –77.0)	70.0 (59.0 - 85.0)	65.0 (55-75)	
Gender				0.815
Male	11 (61.1%)	6 (50%)	9 (60%)	
Female	7 (38.9%)	6 (50%)	6 (40%)	
Years of education (mean ± SD)	13.72 ± 2.52	11.25 ± 3.05	12.80±1.65	0.052
MoCA score (mean ± SD)	27.67 ± 1.19[a]	15.31 ± 7.9[b]	28.47±1.59[a]	**<0.0001***
BMI (mean ± SD)	27.21 ± 3.88	25.88 ± 2.26	26.85± 3.33	0.693
Hypertension	8 (44.5%)	8 (66.7%)	7 (46,7%)	0.449
Hyperlipidemia	7 (41.7%)[a]	5 (38.9%)[a]	0 (0%)[b]	**0.016***
Diabetes mellitus	3 (16.7%)	5 (41.7%)	1 (6.7%)	0.070
Physical activity	12 (66.7%)[a]	7 (58.3%)[a]	15 (100%)[b]	**0.023***
History of smoking	8 (44.5%)	4 (33.3%)	9 (60%)	0.374
Coffee consumption	12 (66.7%)[a]	3 (25%)[a]	0 (0%)[b]	**0.0002***
Played music	3 (16.7%)	2 (16.7%)	0 (0%)	0.2451

SD - standard deviation; Physical activity = walking ≥ 30 min at least 5 days per week; History of smoking = current or former smokers; Coffee consumption = consumption of 3 or more cups daily; Played music = practicing of any kind of music (playing an instrument, singing, dancing), currently or previously in life; * Statistically significant difference. Superscript letters denote which group is significantly different.

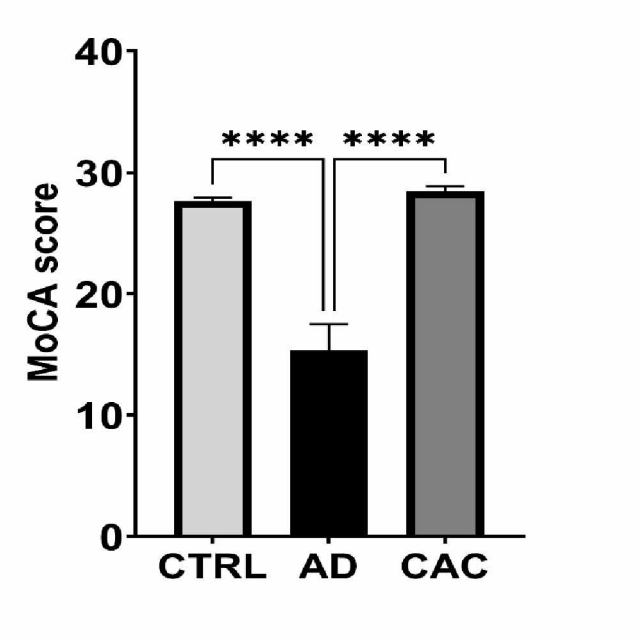


Figure 12. Comparison of the MoCA score among the examined groups. CTRL, control group; AD, patients with Alzheimer's disease. CAC, patients with colorectal adenocarcinoma; * $p < 0.05$, ** $p < 0.01$.

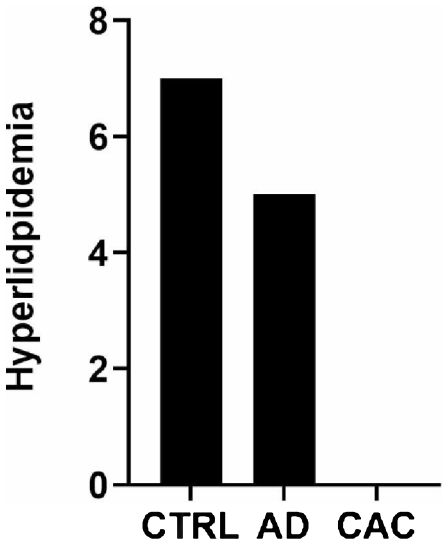


Figure 13. Comparison of the presence of hyperlipidemia among the examined groups. CTRL, control group; AD, patients with Alzheimer's disease. CAC, patients with colorectal adenocarcinoma; Overall $p = 0.016$; Statistically significant difference was found between CTRL and CAC patients, ($p = 0.009$), as well as between CAC and AD patients ($p = 0.009$). There was no statistical significance between CTRL and AD patients.

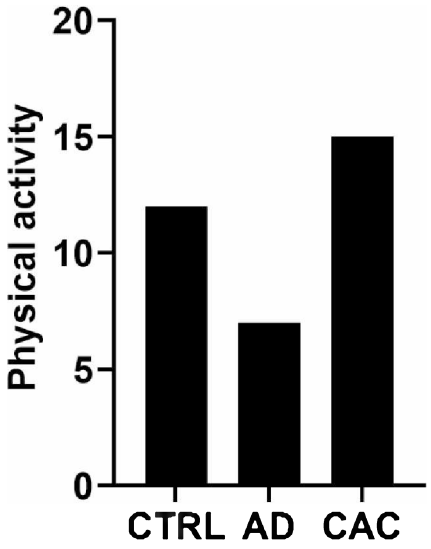


Figure 14. Comparison of the frequency of regular physical activity among the examined groups. CTRL, control group; AD, patients with Alzheimer's disease. CAC, patients with colorectal adenocarcinoma; Overall $p = 0.023$; Statistically significant difference was found between CTRL and CAC patients ($p = 0.021$), as well as between CAC and AD patients ($p = 0.009$). Significance in the frequency of regular physical activity was not found between CTRL and AD patients.

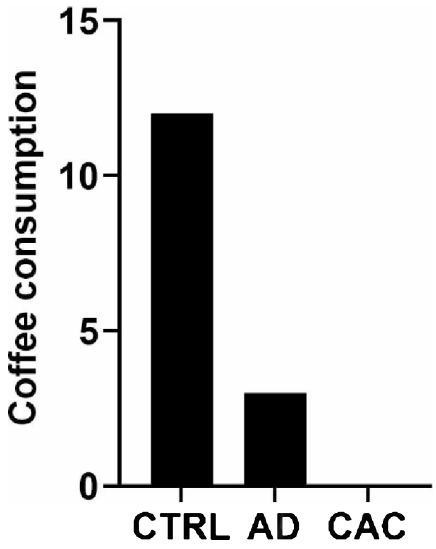


Figure 15. Comparison of coffee consumption among the examined groups. CTRL, control group; AD, patients with Alzheimer's disease. CAC, patients with colorectal adenocarcinoma; Overall $p = 0.0002$; Statistically significant difference was found between CTRL and CAC patients, ($p < 0.0001$). The difference in the amount of coffee consumed, was not significant between patients with AD and control subjects, as well as between AD and CAC groups.

4.3.2. Inverse expression level of miR-101 between AD and CAC patients

In order to test the hypothesis of inverse correlation of selected miRNAs in cancer and AD, the expression levels of specific circulatory miRNAs involved in the pathogenesis of both diseases have been determined in the CTRL, AD and CAC groups by using qRT-PCR. Statistical analysis did not show a difference in the expression of miR-29a, miR-125b, miR-146a and miR-155 among the examined groups ($p = 0.116$, $p = 0.336$, $p = 0.237$, $p = 0.152$ respectively, data not shown).

However, the expression level of miR-101 was significantly different among the groups. The mean expression values of this miRNA for each group showed the expected trend - expression values for the CAC and AD groups were on the opposite sides of the range, while the expression level of the CTRL group was in the middle of the range. Furthermore, miR-101 expression level was significantly higher in AD compared to CAC group (CTRL vs. AD vs. CAC (mean ± SD) = 1.17 ± 0.74 vs.1.57 ± 0.89 vs. 0.83 ± 0.48, $p = 0.032$. However, the CTRL group was not significantly different neither from AD or CAC group (Figure 16).

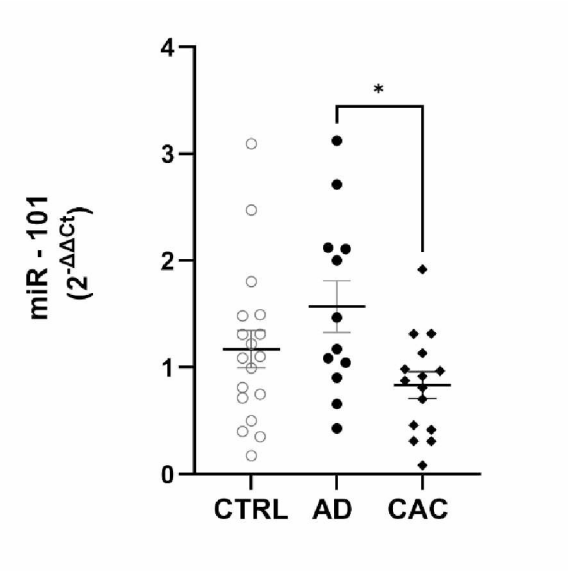
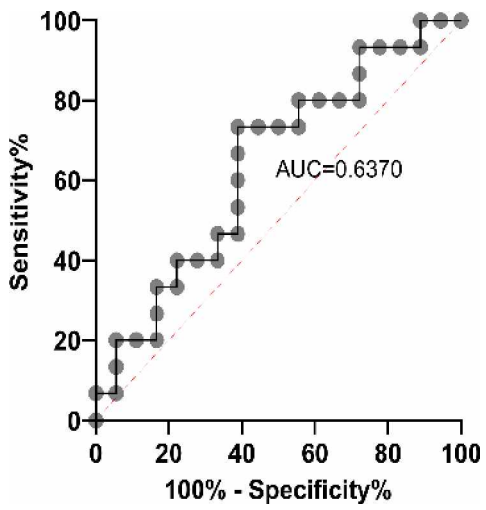
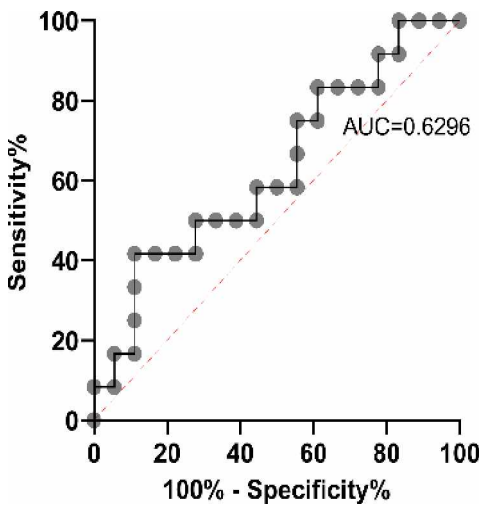


Figure 16. Comparison of the circulatory miR-101 expression levels among CTRL, AD and CAC groups. CTRL, healthy control group; AD, patients with Alzheimer's disease; CAC, participants with colorectal adenocarcinoma; * $p < 0.05$.

To further illustrate the molecular relationship of AD and CAC groups with respect to the general population, that is, to show inverse trends of miR-101 expression between AD and CAC, ROC curve analysis was performed (Figure 17 A and B). For the miR-101 expression values of CTRL and CAC groups, AUC was 0.637 (95% CTRL, 0.445 to 0.828), with 66.67% sensitivity and 61.11% specificity, therefore, ROC curve analysis showed that on average, a CAC patient will have lower expression value of miR-101 than 64% of the controls. For the data on miR-101 expression in participants with CTRL and AD, AUC was 0.629 (95% AD, 0.422 to 0.836), with 66.67% sensitivity and 66.67% specificity, indicating that an AD patient will have upregulated miR-101 compared to 63% of people in the general population. In the absence of significant differences of AD and CAC with respect to CTRL, the results of ROC curve analysis certainly do not demonstrate its diagnostic potential. Nevertheless, they provide the quality score which describes the performance of this AD - CAC inverse correlation model based on miR-101 expression, as a good one. Eventually, the results of ROC curve analysis suggest that the potential of this model to improve the understanding of the inverse relationship between AD and CAC would be even more highlighted if explored on a larger sample size.



A.



B.

Figure 17. Receiver operating characteristic (ROC) curve analysis for miR-101. **A.** ROC for miR-101 in CAC and CTRL groups; **B.** ROC for miR-101 in CTRL and AD groups; CTRL, healthy control patients; CAC, patients with colorectal adenocarcinoma; AD, patients with Alzheimer's disease.

Taking into account the heterogeneity of the AD group, the inverse relationship with CAC was examined with respect to EAD and AAD subgroups. It was found that the miR-101 has decreasing trend of expression, with the progression of AD towards the later stage (EAD vs. AAD, mean ± SD = 1.722 ± 0.945 vs. 1.354 ± 0,705). Consequently, compared among CTRL, EAD and CAC groups, miR-101 expression level showed significantly higher values in EAD compared to CAC (CTRL vs. EAD vs. CAC, mean ± SD = 1.17 ± 0.739 vs. 1.72 ± 0.945 vs. 0.83 ± 0.479, p = 0.028;). Eventually, this finding demonstrates that patients with AAD do not contribute to the significant difference observed between AD and CAC groups (Figures 18 and 19).

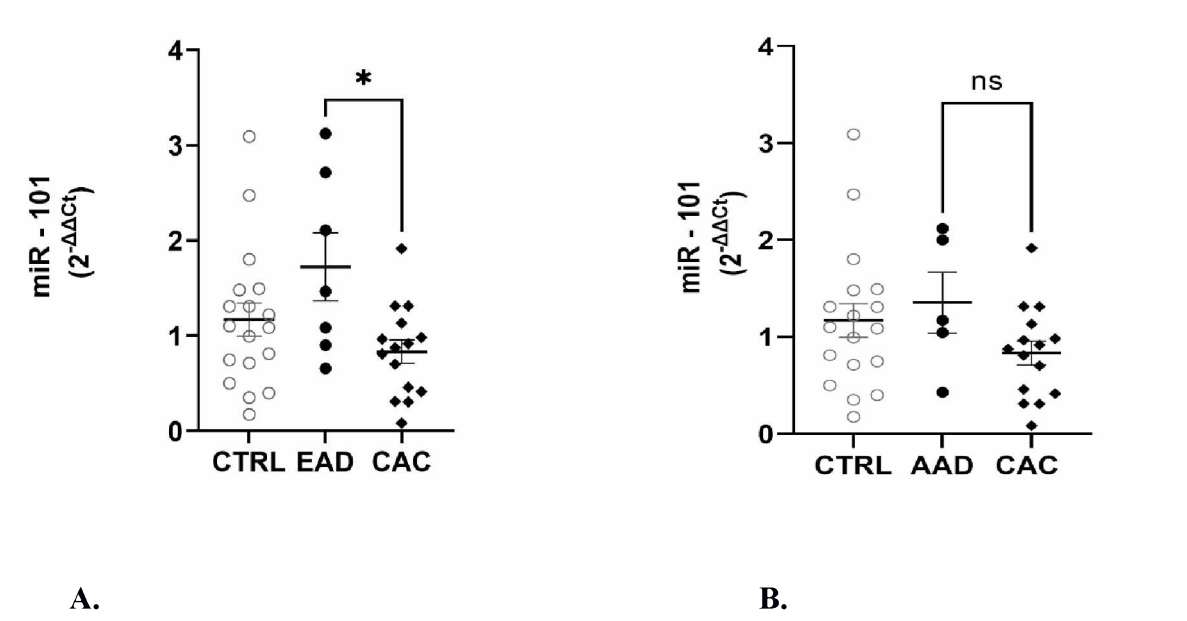


Figure 18. Comparison of the circulatory miR-101 expression levels among each AD subgroup, CTRL, CAC groups. **A**. MiR-101 expression level was significantly different between EAD and CAC groups; **B.** MiR-101 expression level was not significantly different among AAD, CTRL and CAC groups;

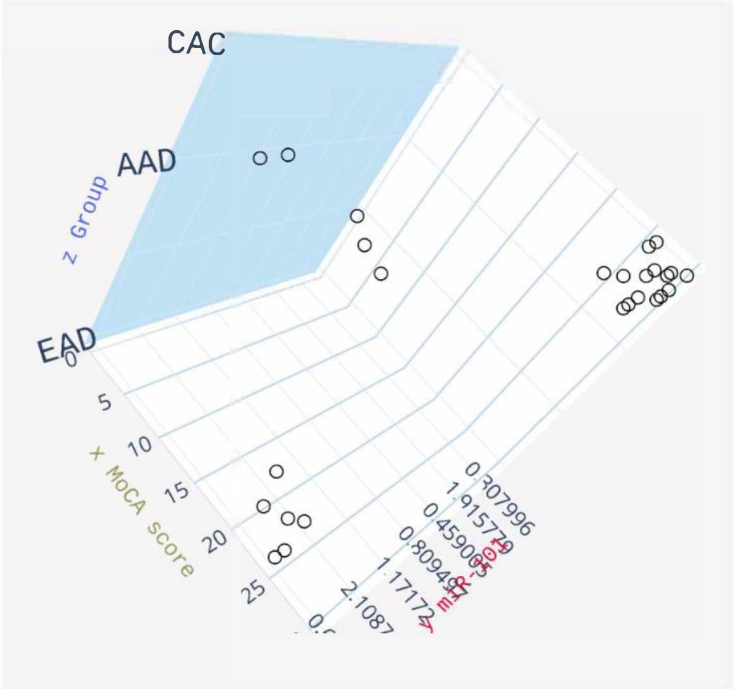


Figure 19. MiR-101 expression level as a function of MoCA score presented in EAD, AAD and CAC as a 3D scatter plot. With the progression of AD towards lower MoCA scores, there is a decrease in miR-101 expression level; based on miR-101 expression, an inverse correlation is detected between EAD and CAC. EAD, patients in the early phase of AD; AAD, patients in the advanced AD; CAC, patients with colorectal adenocarcinoma; * $p <$ 0.05.

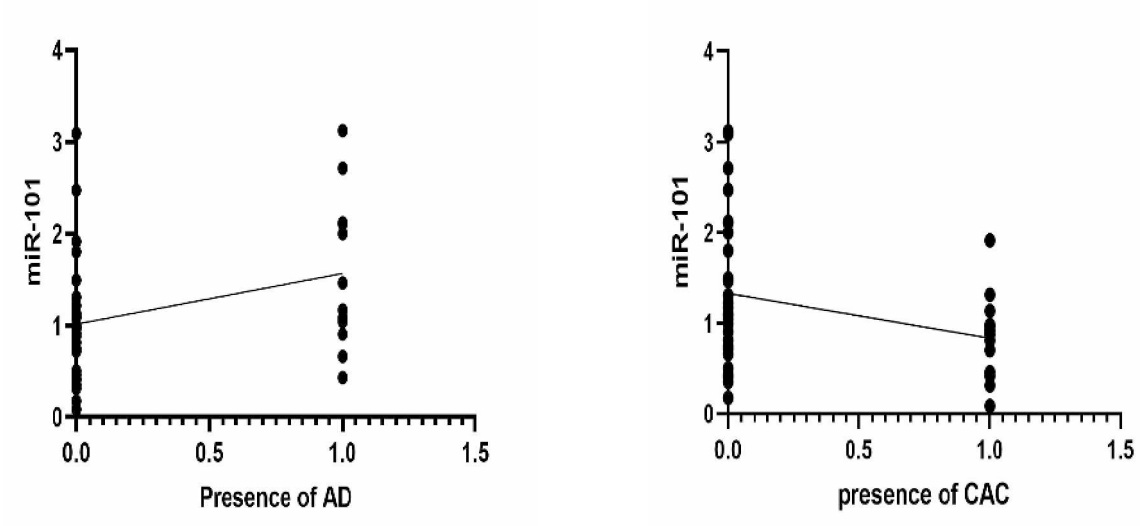
4.3.3. Potential impact of demographic and clinical variables on miRNA expression level in the examined groups

In order to assess the impact of demographic and clinical characteristics of the examinees on the expression level of miR-101, multiple linear regression analysis was performed on the results from **all the study participants, grouped together.** The following covariates were examined in that model: age, gender, years of education, BMI, hypertension, hyperlipidemia, diabetes mellitus, physical activity, history of smoking, coffee consumption, playing music.

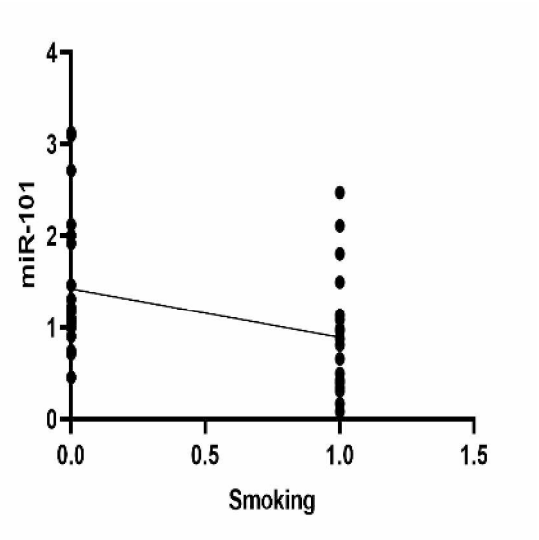
It is important to state, that although variables such as coffee consumption, physical activity and hyperlipidemia were not uniformly distributed among the groups (Table 9), multiple linear regression analysis showed that they did not contribute significantly to the change in the expression of miR-101. The same was true for other variables that were examined in this model, with the exception of the history of smoking.

The results of Pearson's correlation showed that the history of smoking is negatively correlated with the expression of miR-101 ($p = 0.006$).

When the expression levels of miR-101 from all 3 groups, were analyzed together with simple linear regression, it showed that each variable - smoking, presence of AD or presence of CAC, correlates with the expression of miR-101 (Figure 20A - C).



A. **B.**

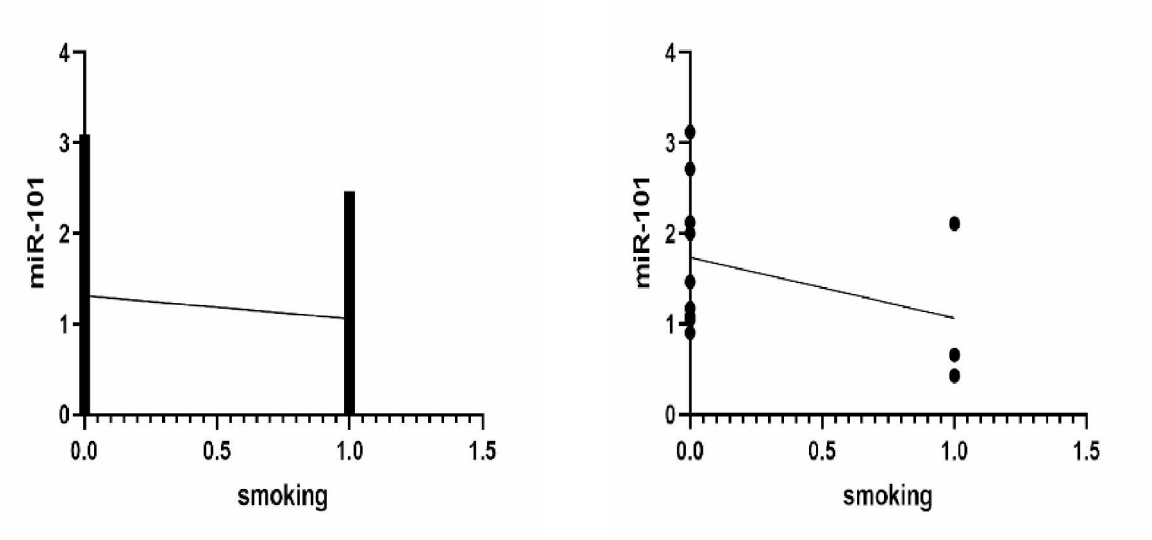


C.

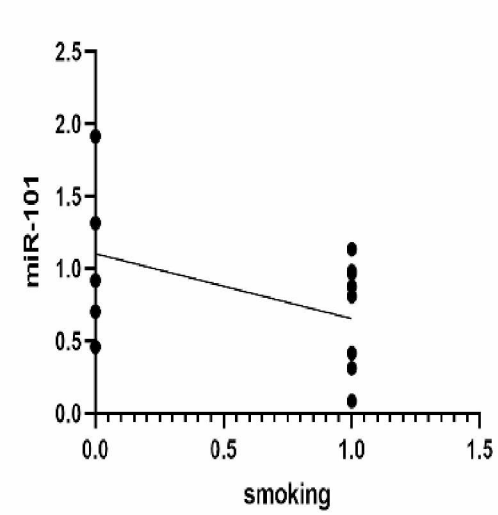
Figure 20. Simple linear regression analysis of the influence of three significant variables on miR-101 expression level. **A**. Influence of AD on the expression level of miR-101, $p = 0.025$, slope = 0.5513, 95% CI = 0.07412 to 1.028; **B**. Influence of CAC on miR-101 expression, p = 0.031, slope = - 0.4958, 95% CI = -0.9457 to – 0.04595; **C.** Influence of smoking, p = 0.014, slope = -0.5298, 95% CI = - 0.9471 to – 0.1125.

The last analysis and figure confirm the significance and demonstrate the way these three variables influence miR-101 expression, suggesting that smoking might bolster the decrease of miR-101 in patients with CAC.

Therefore, it was needed to determine if smoking habits contribute to the variability of miR-101 expression in **each study group separately**, in order to understand if it significantly influences the expression level of miR-101 in AD and CAC patients. Therefore, the impact of smoking on each study group was assessed through a simple linear regression model (Figure 21).



A. **B.**



C.

Figure 21. Influence of smoking habits on miR-101 expression level in each study group. **A**. CTRL group; **B.** AD group; **C.** CAC group; $p = 0.487$

According to the overall results, the possibility of synergistic CAC and tobacco effect on the downregulation of miR-101 should not be ignored. Together with the well-known fact that

cigarette consumption represents one of the major risk factors for CAC, it certainly deserves further attention and consideration of its possible significant effect on miR-101 regulation in the general population. However, when each study group was specifically considered in the context of that influence, no significance was found (Figure 21). This observation is even more important in light of the fact that there were no differences among CTRL, AD and CAC groups regarding smoking habits (Table 9). Thus, we understand that smoking did not significantly affect the observed difference in the miR-101 expression level between AD and CAC patients.

5. DISCUSSION

The main findings of the presented research were twofold. The first part of the results presents the examination of the first two hypotheses, regarding the potential significance of studied miRNAs in AD. It showed that in participants whose performance on the MoCA test indicated they might have MCI, expression levels of miR-146a and miR-155 are significantly increased, compared to the healthy individuals and AD patients. The second part of the research examined the third hypothesis, regarding AD-CAC inverse relationship. This part of the results showed an inverse expression level of miR-101 between AD and CAC patients. The significance of these findings in light of current knowledge on this subject will be discussed in more detail in two separate sections below. The first part of the results is discussed in sections 5.1. and 5.2., while sections 5.3. and 5.4. discuss the second part of the results.

5.1. Significance of changes in the expression level of miRNAs in the examinees of different cognitive status

Descriptive statistical analysis of **demographic and clinical factors** showed that AD, LP-MoCA and CTRL groups were homogeneous (Table 4). In addition, multiple linear regression tests did not detect any impact of demographic and clinical factors on the expression level of miRNAs. (data not shown). These facts increased our confidence in the

credibility of the results of the molecular part of the investigation, which was the essence of this research. On the other hand, the presented study showed that the AD group of patients did not have a higher prevalence of variables that represent risk factors for AD (Table 4). The main potential reason for this could be the relatively small size of the study sample.

Unexpectedly, current research indicated that **the higher the level of the patient's education, the longer it took to diagnose AD** (r = 0.6060, p = 0.036; Figure 5). According to the literature, a higher level of education is associated with better CR (110-113). This led us to conclude, that better CR might imply a higher level of tolerance of CI manifestations. In other words, it might be that these individuals, who should have better performance regarding a variety of tasks, did not have a significant disruption in life activities for a longer period of time, and therefore, they were not compelled to visit a doctor. However, although better CR has been shown to reduce the risk of AD and delay the onset of CI (114,115), it could not explain the longer time to diagnosis. Namely, such patients, once they manifest CI, often demonstrate faster further cognitive decline (343,344). Therefore, it is probable that more educated people are aware of the potential diagnosis, but delay medical consultation, due to a fear of stigmatization. Similar evidence emerges from the recent study, which has shown reluctance among Americans to come for a medical consultation, after noticing symptoms of CI (1). In addition, the growing body of evidence shows that people with dementia experience significant stigmatization (229,345), across a range of layman and professional populations (345). Therefore, this negative behavioral model and ignoring the signs of cognitive decline may stem from the fear of stigmatization and could represent the explanation of the presented result.

As a control group, this study included volunteers who did not report SCD, but interestingly, **based on MoCA scores, 33.3% of the apparently healthy subjects had lower cognitive performance and belonged to the MCI category** (Table 5). **Neurological and laboratory findings could not explain their neuropsychological performance. However, in this LP-MoCA group of patients, the two circulatory miRNAs, miR-146a and miR-155, were upregulated** compared to the control group of patients. The expression level of miR-146a was also significantly higher in LP-MoCA compared to the AD group (Figure 8A and B). After we divided the AD group into early and advanced AD, the results indicated that the

EAD group also followed this interesting expression pattern for both, miR-146a and miR-155, the same as the heterogeneous AD group. (Figure 10A and B). However, the difference in the expression level of miR-146a and miR-155 between EAD and AAD was not statistically significant. Finally, ROC curve analyses suggested that these miRNAs could serve as non-invasive biomarkers in screening for early cognitive impairment (Figure 9).

SCD is known as a phenomenon of self-experienced cognitive decline that may represent the first manifestation of AD when objective impairment in cognition is still not present (155,156). MCI according to the neuropsychological screening, in healthy subjects who did not report SCD, has not been clinically recognized so far, to the best of our knowledge. Many studies showed the importance of SCD for early prediction of the development of clinically manifested AD (30,155,156,346,347). Moreover, neuroimaging techniques revealed distinct brain alterations related to the symptoms of SCD (347,348). However, there are some critical points in the process of SCD evaluation that might be too subjective, affected by the individual cultural background and susceptible to the influence of various social factors and interpersonal relations at the moment of evaluation (156,157). Our decision to use simple, open-type questions for SCD evaluation instead of structured questionnaires was in part driven by these facts. Results of neuropsychological screening tests used, MoCA and MMSE, were in correlation ($r = 0.725$; $p < 0.01$, Figure 6), but MoCA proved to be more sensitive, which is consistent with previously published data (340). Lower cognitive performance would not have been discovered in 22.2% of the examinees, had they been evaluated by MMSE only (Table 5). Moreover, if the evaluation of SCD was not followed by an objective assessment, none of the participants with mild cognitive impairment would have been identified. Thus, our results certainly raise a question of the reliability of subjective comprehension of cognitive functioning and emphasize the significance of objective neuropsychological assessment. The absence of cognitive complaints in the examinees with the worse neuropsychological performance might be explained by our results. Namely, the MoCA score of the volunteers within the MCI range is positively correlated with the years of their education ($r = 0.491$; $p < 0.05$, Figure 7). This suggests that lower education might be the underlying cause of inadequate comprehension of the importance of changes in cognition, which might be a critical sign of impaired health.

In the further search for the causes of detected MoCA performance in MCI range and in order to rule out other causes of cognitive decline, it was first noted that there was no significant difference in age among the groups (Table 4). History of other diseases, brain injury, and the list of medications were reviewed. The participants were also checked for vitamin B12 deficiency, thyroid dysfunction, anemia, and other acute or chronic conditions that could cause MCI (349,350). Finally, no pathological findings were observed either during the neurological examination or the geriatric depression scale test. However, it is worth noting that the extent of neurological evaluation in the presented study was limited to non-invasive and inexpensive tests and also determined by the fact that AD patients have been retrospectively recruited.

Interestingly, the LP-MoCA group had significantly higher levels of miR-146a and miR-155 expression, in comparison to the healthy control subjects (Fig 8A and B). Various studies conducted in human or animal models and cell cultures over the last ten years unambiguously demonstrated the involvement and significance of miR-146a and miR-155 in the pathogenesis of AD (300-302,308-310,351). MiRNA-146a was among the first miRNAs found to be highly expressed in the AD brain, specifically in anatomical regions affected by the disease but not in the control regions of the same brain (299). Authors of the recent bioinformatics study, who reviewed and extracted data from miR-TarBase on the currently known AD-associated miRNAs, and who formed the miRNA-target network, identified miR-146a as one of the central molecules in the pathogenesis of AD with a biomarker potential (352). As for miR-155, its expression was found to be increased in AD rats, and its inhibition improved impaired memory in this animal model (308).

When considering the continuum of clinical presentation of AD, it is of special interest to identify miRNA signature patterns of the MCI stage, since it is estimated that up to 22% of individuals clinically defined as MCI, progress to AD within one year (353). More importantly, in this phase, there is a possibility to apply disease-modifying therapy and postpone the onset of dementia (68,165). Circulatory miR-146a is known to be significantly up-regulated in patients with MCI who later develop AD, compared to those who do not convert to AD (351). Also, a higher miR-146 expression level was found in +*APOE* - ε4 carriers, and this finding correlated with neuroimaging hallmarks of AD, as well as increased

CSF Aβ42 concentration (351). An interaction among MCI-associated genes and miR-155 was emphasized in the study by Strafella et al, which also found that miR-146 and miR-155 signaling pathways significantly interact in a pathophysiological cascade of AD and other neurodegenerative diseases (354). Taken together, these findings suggest that AD could be an underlying cause of the accidentally discovered low MoCA performance in the MCI range in volunteers in our study, who had increased expression of miR-146a and miR-155, compared to the control group.

A closer understanding of miR-146a and -155 involvement in particular pathophysiological pathways of AD further explains the significance of their increased expression level in the LP-MoCA group. MiR-146a is known for its importance in modulating the innate immune response and inflammatory events in brain cells (300-302). It has been recently proposed as highly significant in the neuroinflammatory mechanisms of AD (351,354-356). For example, in primary human neuronal-glial cell co-cultures, miR-146a transcription was found to be induced by certain stress factors, such as the pro-inflammatory cytokine interleukin 1 (IL-1), known to be elevated in AD brain (358). More recent research on inflammatory processes in AD also revealed the significance of miR-155 in these pathways (308-310,354,355). This miRNA was shown to be early and strongly up-regulated in a 12-month triple transgenic mouse AD model (309), but also in Aβ-activated microglia and astrocytes, contributing to the production of inflammatory mediators such as IL-6 and IFN-b, inducing the decrease of activity of cytokine signaling suppressor (358,359). Moreover, these studies revealed not only the involvement of miR-146a and miR-155 in neuroinflammatory AD pathways but also their interactive points in that cascade (354). On the other hand, it is well established that neuroinflammation contributes to AD pathogenesis (69-85,88-94), and there is evidence that strongly suggests that it is an initial and vital component in the AD pathophysiological cascade (66,92,360,361). In an animal model, activation of microglia, which are key mediators of neuroinflammation among the innate immune cells, has been observed at the pre-plaque stage of AD (360). Also, increased microglial activation has been detected in people with MCI, in the absence of amyloid tracer uptake (92,361). All these data go in favor of the hypothesis that an increase in expression levels of inflamma – miR-146a and -miR-

155 in LP-MoCA subjects could be explained by their involvement in inflammatory pathways, characteristic of the early phase of AD pathophysiological events.

The presented results also showed that the expression levels of miR-146a and miR-155 were not statistically different between control and AD subjects and interestingly, miR-146a was still up-regulated in LP-MoCA compared to the AD group. More thorough insight into neuroinflammatory AD events and engagement of miR-146a and miR-155 in those pathways, could offer an explanation for such a result. Although essentially defensive, the immune response can cause harmful consequences if it is induced too strongly or for too long (362,363). Thus, at some point in time, there is an activation of homeostatic mechanisms to limit destructive inflammatory events in AD (364,365). Published data clearly indicate that miR-146a also has a role in the suppression of pathological neuroinflammatory response in AD. Primarily induced by pro-inflammatory cytokines (357,366,367), miR-146a in turn down-regulates proteins in overactive neuroinflammatory signaling pathways, contributing to their limitation (355). Consequently, it is possible that this negative regulatory feedback mechanism ultimately ends with decreased expression of miR-146a. This consideration is supported by the research on primary neuronal cultures or neuroblastoma cell lines bearing Swedish mutation as AD cell models, which showed that miR-155 and miR-146a were highly expressed in microglia, responding to Aβ as a stress-related factor, with a more prominent role of miR-155, which is found to be responsible for microglia polarization to pro-inflammatory M1 phenotype. Moreover, a subsequent increase in inflammatory cytokines was followed by a reduction of miR-146a expression, while miR-155 upregulation persisted (368). Another study by the same authors (369) showed that the presence of Aβ in different assembly states interacts with microglia leading to an inflammation cascade in young cells. This response was lost in aged cells, suggesting a differential response along the progression of AD. A temporal discrepancy of miR-146 and miR-155 expression during an inflammatory response was confirmed in animal models as well (370). Increased expression of miR-155 induced overactive acute, but also chronic inflammation, even in miR-146a-deficient mice (370). These results are in line with our findings of miR-146a and miR-155 expression levels in a clinical context. Significant miR-146a upregulation in LP-MoCA compared to the control group corresponds with its dominant role early in the disease process, through the

initiation of inflammatory cascade and interaction with mediators of inflammation. Normalization back to control levels in the AD group probably reflects suppression of miR-146a by homeostatic, anti-inflammatory mechanisms, characteristic of the chronic stage. On the other hand, miR-155 expression implicates its persistent activity, as a reflection of continuous, chronic, although self-limiting inflammation and continuous microglial engagement in that process.

However, other studies showed that disease progression in AD mouse models was followed by increased miR-146a expression in brain tissue (356), and it was observed in the same model *ex vivo*, that the density of plaques and synaptic pathology were in correlation with miR-146a expression. This contrary observation could be caused by the different methodological approach, that is, potentially different timelines of the expression measured in the brain tissue, compared to the levels of circulatory miRNAs. Similarly, Lukiw et al. found that miR-146a levels measured in the neocortex and limbic system increased, as the severity of AD advanced (357).

Our results also show that when analyzed together, the **specific expression patterns of miR-146a and miR-155 were able to differentiate the LP-MoCA group from the control as well as from the AD group of participants** (Figure 11). AUC value of 0.8642 for miR-146a, with 77.8% sensitivity and 94.4% specificity (Figure 9A), and AUC value of 0.7654 for miR-155, with 66.7% sensitivity and 88.9% specificity (Figure 9C), clearly suggests their potential diagnostic significance in screening for MCI. More research is needed to determine if these miRNAs could be used for screening the general population on early AD, stratifying those for further procedures and potential confirmation of diagnosis.

Finally, our **results on miRNA expression levels among CTRL, LP-MoCA and EAD groups, additionally support their significance in screening for very early cognitive impairment** (Figures 10A and B). Apart from the potential of these miRNAs to distinguish people with cognitive impairment, but still feel subjectively healthy, from healthy population, they indicate that miR-146a could differentiate very early cognitive impairment from heterogeneous AD population, including the early stage of established AD. This highlights the potential of miR-146 to be used as a screening test for individuals with early cognitive impairment who are at risk of developing clinical manifestations of AD. More importantly,

this part of the population would thus be non-invasively selected for further diagnostic evaluation, in order to potentially establish biological confirmation of AD very early, and therefore, be suitable for disease-modifying therapy. In addition, there was no significant difference in the expression levels of miR-146a and miR-155 between the EAD and AAD groups, which implies a stable expression level of these miRNAs throughout the different cognitive stages of established AD. This fact also supports the interpretation of miR-146a potential to be used as a screening instrument for detection of individuals with cognitive decline who are at risk for AD, but still do not fulfill clinical criteria for AD diagnosis.

The overall, presented **first part of the results, does not support the first hypothesis**. The expression levels of the examined miRNAs did not correlate with the level of cognitive decline in AD patients. Moreover, the same can be concluded when considering the LP-MoCA group, which was unexpectedly formed during the research process. However, precisely the specific expression pattern of the investigated miRNAs that was detected among the study groups and did not correlate with the level of cognitive impairment, **confirms the second hypothesis.** As previously discussed, miR-146a and miR-155 isolated from the plasma, could identify people with cognitive decline who are at risk for AD, thus serving as screening tools for the general population.

5.2. The analysis of the miR-146a and miR-155 target genes supports the results of the present research

A systematic analysis of miR-146a and miR-155 target genes through the STRING database (371) offered additional confirmation and valuable source of information, on their roles in the pathogenesis of Alzheimer's disease. Namely, detailed functional annotation and visualization of the selected genes gives invaluable insight into the complexity of miR-146a and miR-155 domains of action.

One hundred miR-146a target genes were selected by this systemic analysis approach. This selection was supported by strong evidence determined by Reporter gene assay, Western blot, or qRT-PCR (Attachment 6). Among them, using the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, 9 genes that mediate known AD signaling pathways

were identified (Table 6). Considering the dominance of AD neuroinflammatory theory, involvement of miR-146a in inflammatory cascades, as well as in the context of the results of the present research, an additional analysis of AD-related genes was done, aimed at the selection of genes involved in immune and inflammatory biological processes. In total, as many as 7 out of 9 genes involved in AD pathogenesis were detected as mediators of immune and inflammatory response (Table 6). This finding supports the results of the presented research, therefore, an in-depth analysis of the functional context of those 7 genes was conducted. The results indicate, that 4 genes: tumor necrosis factor (TNF), interleukin-6 (IL-6), prostaglandin-endoperoxide synthase 2 (PTGS2) and FAS-associated via death domain (FADD) encode proteins responsible for the early immune and inflammatory response (371). One more gene-nuclear factor NF-kappa-B p105 subunit (NFKB1), was identified as the mediator of the cell response to the endogenous stimulus, which also implies an early reaction to the pathological environment (371). These database search results further reinforce our conclusions. Namely, the up-regulation of miR-146a, detected in the LP-MoCA group (Figure 9A), was attributed to its role in the initiation of the inflammatory cascade in AD, which was confirmed by the systematic investigation of its target genes (Table 6). Furthermore, we hypothesized that by the negative regulation of mediators of the destructive initial acute inflammatory response, miR-146a upregulation is a self-limiting process. Consequently, as their activity decreases, miR-146a downregulation probably occurs through a negative feedback mechanism, which explains the normalization of the miR-146a expression level in the AD group (Figure 8A). The analysis of the involvement of miR-146a targets in a chronic inflammatory response has detected only one gene (371), which further supports the hypothesis related to the miR-146a expression level fluctuation in LP-MoCA and AD groups that emerged from the present study.

After the selection of 260 miR-155 target genes, supported by the strong evidence (Attachment 7), further analysis through the STRING database initially did not identify AD-related genes, as for the miR-146a. However, 32 genes related to the CNS diseases were initially detected and subsequently out of those 32 genes, 10 target genes responsible for the neurodegenerative diseases were identified (Attachment 7). Evidence of an association with AD was found in published papers for 8 out of 10 genes related to neurodegenerative

diseases: macrophage colony-stimulating factor 1 receptor (CSF1R), T-cell surface glycoprotein CD4 (CD4), interleukin 1B (IL1B), FADD, phosphatase domain containing, paladin 1 (PALD1), phosphatidylinositol-binding clathrin assembly protein (PICALM), brain-derived neurotrophic factor (BDNF) and 14-3-3 protein zeta/delta (YWHAZ) (Table 7) (372-382). In accordance with the context of the present research, the involvement of the selected genes in immune and inflammatory biological processes was further analyzed and identified through the STRING database. Three genes – FADD, CSF1R, and CD4, are denoted as significant in the activation of immune response and IL1B as the proinflammatory gene (371). The published research confirms that all of them are strongly related to the changes in the immunological microenvironment, microglial dysregulation and neurodegeneration–specific microglial profiles (373-377). The other 5 genes were found to be involved in the regulation or modification of APP and tau processing (378), maintenance of synaptic plasticity (379), crosstalk for AD-PD pathogenesis (380) or they were simply detected as differentially expressed in AD but the precise mechanisms of their contribution in this pathology are still not elucidated (381,382). Finally, the implications of the analyses of miR-155 target genes and the context of the present research, seem to be in agreement. Significant up-regulation of miR-155 was detected in the LP-MoCA group compared to the CTRL, however, its expression level did not differ between the LP-MoCA and AD groups (Figure 8B). Namely, miR-155 up-regulation in the LP-MoCA group might be explained by the involvement of its target genes in the activation of microglia and the creation of a specific immunological microenvironment. However, it has been demonstrated that microglial engagement persists during the chronic stage of the disease, supporting the result of the stable miR-155 expression level with the progression of cognitive pathology.

Next, in order to investigate miR-146a and miR-155 overlapping roles in AD, a number of target genes shared by these two miRNAs were selected (Table 7). Only one common target related to AD pathology was identified – FADD gene, which encodes a protein involved in immune response and which has been already discussed. Therefore, these two miRNAs are involved in pathophysiological cascades of AD, mainly targeting different genes. However, the immune and inflammatory signaling pathways represent the common framework of their regulatory actions, which are mostly of different types and dynamics. This aligns with the

discrepancy of their expression pattern in the present research, as previously discussed. In addition, a significant number of miR-155 target genes are involved in the hallmark AD pathophysiological cascades, such as the regulation of APP and tau accumulation or preservation of synaptic plasticity (378,379), which further supports stable miR-155 expression level, once it was upregulated early in the pathological process.

5.3. Relationship between CTRL, AD and CAC groups and the role of clinical variables

In the second part of the research, we compared CTRL, AD and CAC groups. Although the groups were balanced regarding the demographic factors (Table 9), an unequal distribution of clinical variables with potentially important implications was detected, and this is discussed in the text below.

5.3.1. Hyperlipidemia

Hyperlipidemia has been identified as a significant risk factor for CAC (383). It has been demonstrated that increased levels of low-density lipoprotein cholesterol and triglycerides contribute to the activation of the oncogenic intracellular signaling cascades (384,385). The latest meta-analysis which explored the significance of non-genetic risk factors for early-onset colorectal cancer, indicated a significant association between hyperlipidemia and an increased risk of developing CAC in adults under 50 years of age (386). Moreover, the results of this study suggested that this comorbidity could be an even stronger risk factor for early-onset colorectal cancer than CAC in general (386). However, in our study none of the CAC patients had hyperlipidemia, so this comorbidity was significantly more prevalent in patients with AD as well as in healthy individuals, compared to the CAC group (Figure 13). The reason for this observation might be related to the disease itself - decreased appetite and significant weight loss are one of the early and most common manifestations of many types of cancer. There is a vast amount of data on the significance of hyperlipidemia and metabolic syndrome for the occurrence of AD (216-220). However, higher blood lipid levels were not more prevalent among AD patients compared to the healthy control group (Figure 13). Thus,

although the literature data suggest the importance of lipid disorder in AD pathogenesis, it cannot be confirmed that it significantly contributed to the disease occurrence, at least not in the sample size presented here.

5.3.2. Coffee consumption

Our data further show that coffee consumption was significantly lower in the CAC group compared to the other two groups ($p < 0.01$, Figure 15). Numerous data from the literature indicate that regular daily intake of moderate amounts of caffeine, may decrease the risk of AD and CAC (387-393). European Food Safety Authority recommends that caffeine intake should be a maximum of 400mg per day (394). However, some of the more recent research has demonstrated that a dose of 500mg of caffeine per day would be more beneficial in the prevention of cancer and AD (395,396). Coffee drinking habits in Montenegro, include by far the most common consumption of Turkish coffee, which has over 100 mg of caffeine per cup (397). Considering these data as well as coffee drinking habits in Montenegro, 3 or more cups of coffee were considered as potentially significant preventive factors as described in the Results section in more detail (Tables 4 and 9; Figure 15).

It has been shown that as the amount of coffee consumed increases, the risk of CAC decreases (387). Many coffee ingredients, in addition to caffeine, support colon health (398,399), as decaffeinated coffee was also related to lower CAC risk (387,400). The preventive potential of coffee has been attributed to its potential to modulate the gut microbiome, improve bowel function and reduce the synthesis of bile acids through its lipid compounds (398,401,402). More importantly, coffee contains powerful anti-oxidants and anti-mutagens which oppose the process of carcinogenesis (402-404). Numerous literature data have also shown an association between regular intake of moderate amounts of caffeine with better cognitive function and lower AD risk (390-393). Results of the experimental studies on animal models or cell cultures are even more conclusive in this regard. For example, the treatment of mice with moderate doses of caffeine improved their memory (405,406). It has been demonstrated that the treatment of mice with caffeine in a dosage equivalent to human 500mg/d, significantly reduced Aβ deposition (395,396). Cultured neurons exposed to Aβ accumulation were protected from toxic effects by a reduction of intracellular ROS

accumulation and an increase in activity of antioxidant enzymes, which confirmed that caffeine has antioxidant properties, that might also be beneficial in AD (407). Moderate and regular intake of coffee is considered not only useful in delaying or preventing AD but, considering convincing literature data, it is believed that its effects might also have therapeutic significance (393,396).

All of the CAC patients and 75% of AD patients consumed maximum 1-2 cups of coffee, and some did not consume it at all (Figure 13). These results are in line with the reported inverse association of AD and CAC and caffeine intake (387-393,395,396,402,408,409). Over 65% of healthy individuals regularly consumed significant daily amounts of coffee, which further agrees with the reported health benefits of caffeine.

5.3.3. Physical activity

In contrast to the results on coffee consumption, 100% of CAC patients included in this study, reported that they had regular physical activity previously in life. Physical activity of older adults has been particularly defined in the literature: it is moderate, if aerobic physical activity lasts for at least 20 minutes 3 days per week, or more intensive, if aerobic activity is practiced for 30 min daily 5 days a week (410). Patients diagnosed with CAC were significantly more physically active compared to the CTRL and AD examinees (Figure 14). The association between physical activity and CAC has been investigated in over 50 studies (411,412), offering unambiguous and convincing results, that physical activity contributes to the reduction of risk for CAC by about 25% (411-414). Moreover, a larger reduction in CAC risk is likely to be associated with higher doses of physical activity, especially if it is of greater intensity (411,415). Furthermore, this dose-response effect was objectified in one study, estimating that individuals with the highest level of physical activity had a 19% lower risk of CAC compared to those with the lowest physical activity (416). Potential mechanisms of protective effects of physical activity imply its positive influence on the gut microbiome (414,417,418), whose imbalance is considered one of the critical factors for the development of CAC (419). Other identified benefits are related to increased immune response and changes in inflammatory, as well as in insulin-related pathophysiological cascades, which counteract cancer development (414,420). It can be concluded that the results of the present

study do not confirm the preventive potential of regular physical activity on the occurrence of CAC. Considering the data about the stronger influence of higher levels of physical activity on the reduction of CAC risk, one of the possible explanations of such a result may lie in the type of activity reported by the study examinees. Namely, they have indicated walking as a predominant physical activity, therefore it could be associated with a smaller potential for reduction of CAC risk, which cannot counteract the contributing factors. In addition, the relatively small size of the sample examined, as well as the subjective estimation of the level of physical activity, might limit the conclusions that emerge from this study. Eventually, such contradictory data confirm that there are many possible contributing and preventive factors in complex diseases like cancer, which in a unique combination and circumstances in a particular individual may or may not lead to the disease.

Similarly, regular physical activity has been shown to be protective against dementia (421,422). The inverse relationship between the risk of cognitive impairment and a lifestyle involving regular physical activity has been well documented (423). Regular physical activity has been shown to reduce the risk of developing AD by about 45% (424), and those having a low level of physical activity had a 53% higher chance to be diagnosed with AD than the more active individuals (425). One of the first studies that pointed out the importance of physical activity for brain health, found that one year of moderate-intensity exercise, increased the size of the hippocampus as well as spatial memory performances, in healthy elderly populations (426) The latest very interesting study in this field reached the conclusion that six minutes of high-intensity exercise might delay the onset of AD as well as other neurodegenerative diseases (427). The critical beneficial point is the fact, that short intervals of high-intensity training increase 4 to 5 times more circulating BDNF, which promotes neuroplasticity, compared to prolonged light exercise (427). In experimental animal models, exercise was among the environmental factors that reduced Aβ in the brain of the mice (428), through stimulation of angiogenesis, neurogenesis, synaptogenesis, and production of neurotrophic factors (428-431). In the present study, more than half of the patients diagnosed with AD reported being regularly physically active earlier in life, which was not different from the CTRL group (Figure 14). Therefore, this result is not in agreement with the indicated evidence from the literature. However, it has been also documented that improvement of

cognitive function due to aerobic exercise is not always achieved in elderly aged between 60 and 80 years (432), which is exactly the population represented in this study. Nevertheless, in contrast to the CAC group, 42% of AD patients reported having a low level of physical activity previously in life, which could have contributed to the onset of the disease in that part of the AD group. Finally, the small study sample as well as the subjective assessment of the level of physical activity, certainly limit conclusions in that regard.

5.4. Alterations of miRNA levels among CTRL, AD and CAC groups

Among five examined miRNAs in the present study, only the expression levels of miR-101 in examined groups were in agreement with the hypothesis on the inverse relationship of pathogenetic processes between AD and CAC. The mean expression values of miR-101 for the CAC and AD groups were on the opposite sides of the range, while the expression level of the CTRL group was in the middle of this spectrum. Furthermore, miR-101 expression was significantly higher in AD compared to the CAC group (Figure 16). The CTRL group was not significantly different neither from AD or CAC group. However, results of ROC curve analysis indicated that on average a CAC patient will have lower miR-101 expression than 64% of controls, and an AD patient will have upregulated miR-101 expression compared to 63% of people in a healthy general population (Figure 17A and B). This analysis provided the quality score which describes the performance of this AD-CAC inverse correlation model based on miR-101 expression, as a good one. This model was also tested with respect to the disease stage of the AD group. Interestingly, the miR-101 expression level was significantly higher in EAD compared to CAC (Figure 18). However, its expression values decreased with the progression of AD toward the later stage, approaching the level of expression in the CAC group (Figures 18 and 19). Therefore, no significant difference was observed between AAD and CAC groups, indicating that patients with AAD do not contribute to the significant difference observed between AD and CAC groups (Figures 18 and 19). Regarding these results, there are several aspects that need to be understood. In the first place, their significance in the context of AD, and then CAC, so that in the end, we could comprehend

whether the miR-101 opposite expression values in AD and CAC groups in this study reflect an inverse relationship between these diseases.

5.4.1. The role of miR-101 in the pathogenesis of AD

The most important miR-101 physiological role that has been studied the most in the context of AD, is **regulation of *APP* gene expression** (315,433). It is assumed, that based on that role, miR-101 directly mediates the pathogenetic process of AD (315,316,433). *APP* is one of the few dominant autosomal genes, whose alterations are unambiguously associated with AD (29,38,39,52,55,59,102). More than 30 mutations in 18, out of 770 nucleotides of the *APP* gene, as well as its duplication, are responsible for the familial form of the disease (38,39,434-436.). The sporadic disease form, although a multifactorial disorder, is also found to be related to *APP* mutations, particularly when the disease has early onset (434). More recently, mutations of the *APP* regulatory sequence have also been demonstrated, and they probably contribute even more than dominant mutations to the increased risk of developing AD (437,438). A common feature in these alterations is that they all ultimately cause increased expression of *APP* in AD (66,439,440,441,442). In addition, even when the disease is not characterized by *APP* gene alterations, like in the LoAD, *APP* overexpression is found to be induced by other factors. In the neuroinflammatory process, which is probably the substantial driver of AD, excessively released mediators, such as IL-1, TNF-α and IFN-γ, promote *APP* expression, leading to its up-regulation through the joint action (66,91,93,300,439,440). Mitochondrial dysfunction, as one of the pathophysiological aspects involved in AD, was also found to influence *APP* expression and related downstream pathological processes (300,441,442). Therefore, *APP* overexpression seems to be an inevitable part of AD pathogenic mechanisms. The mechanisms of *APP* up-regulation and even the resulting cascades of events related to specific mechanisms are different, but with an unambiguous consequence - they result in **increased production of APP**, together with pathological modifications and dynamics of its metabolism (29-34,52,55,443). APP is a transmembrane glycoprotein, widely represented in many organ systems and abundant in neurons. It breaks down into different fragments, among which, the Aβ cleavage product with 42 amino acids is the most hydrophobic. Aβ has a tendency to aggregate and form

extracellular amyloid plaques, characteristic of AD. Physiologically, APP is degraded subsequently by β and γ secretases, with a subtle representation of the Aβ fraction (56-59). However, in AD, Aβ pathogenic pathway is triggered, mainly due to altered APP metabolism, caused by variations of *APP* or genes that encode key enzymes of this process (29-34,52,59,60). Neuroinflammatory mediators may also directly affect the activity of enzymes responsible for APP metabolism (440). In summary, multiple and often simultaneous signals and mechanisms are involved in the pathogenic APP metabolism, with the *APP* gene overexpression as the most prominent cause, and the consequent increase in Aβ production, up to toxic concentrations. Highlighting the importance of this phenomenon of *APP* up-regulation in AD, the *APP* gene dose hypothesis was established, with *APP* gene inhibition as a central idea in the development of new therapeutic modalities (444). **MiR-101 negatively regulates the expression of the *APP* gene** (315,433,445-447). It interacts with the 3′ UTR of a target mRNA transcript, causing mRNA destabilization and/or translational inhibition (271,272,285-287,313). In accordance with this physiological action of miR-101, other studies demonstrated that blocking of its interaction with *APP* gene leads to up-regulation of *APP* expression (445). Also, it has been shown that inhibition of miR-101 action in the culture of hippocampal neurons resulted in the enhanced pathogenic-amyloidogenic processing of APP (433,446,447). Similarly, HeLa cell transfection with the miR-101 inhibitor resulted in the enhancement of *APP* gene expression, which was previously significantly reduced, by transfection of miR-101 mimic (445). Thus, the reversibility of the miR-101 effect, which has been shown in this study, offers strong evidence and insight into its regulatory potential on *APP* gene expression. Also, it is interesting to mention, that one study shows even a direct effect of miR-101 on APP production, without mediation of mRNA, as the expected mode of miRNA action (315).

Many studies, with diverse methodological settings, are conducted with the aim **to explore miR-101 role in AD** - in AD patients, animal models or cell cultures (316,319,448,449). They are mostly in agreement and indicate that the expression level of this miRNA is reduced in AD (316,448,449). Since this disease is characterized by *APP* gene overexpression, miR-101 down-regulation associated with AD is mainly interpreted as the contributing factor. Namely, it is assumed that lack of its natural, inhibitory influence on *APP* gene, results in its

pathological overexpression in AD with a consequent modification of APP metabolism in the upstream manner, finally leading to the increased Aβ production and its accumulation to the toxic levels. A study showing that miR-101 down-regulation is associated not only with the *APP* gene up-regulation and increased APP metabolism but also with cognitive impairment is considered strong evidence for the significant contributing role of this miRNA in AD. Assessment of the cognitive status of adult mice in this study indicated cognitive decline after the intrahippocampal injection of miR-101 inhibitor (446,447).

In contrast to this literature evidence, **in our study, there is an up-regulatory trend of miR-101 expression level in AD patients,** with respect to the general population and significantly increased miR-101 expression, compared to the CAC population of patients, known to be associated with decreased probability for development of AD. In an attempt to understand this finding, the precise mechanisms of miR-101-*APP* gene associations demonstrated in AD, were investigated. One of the probable explanations represents mutations of *APP* 3'UTR, which is the target place of miRNAs action, thus influencing miRNAs function (450,451). On the other hand, miRNA single nucleotide polymorphism (SNP), mostly in premature forms, cause loss or add biological functions to mature miRNA molecules (287), which has been shown to influence miRNAs expression and function (452,453,454). The relationship between SNPs of miRNAs and AD has been demonstrated as well (453,454). Interestingly, evidence from the literature suggests that these types of alterations may be specific to a specific population (455-457), but this has not been explored in most European regions, including South-East Europe, at least not in the context of AD. So, the question of whether there is a specificity of miR-101 polymorphisms or of its target *APP* gene place, that potentially contribute to different dysregulation of miR-101 found in this study, represents a possible explanation but remains to be explored in the future.

On the other hand, the findings of this study might be explained differently from the currently accepted mainstream thought. Since one of the main hallmarks of AD is an accumulation of Aβ, resulting from the increased, pathogenic APP metabolism (29-34,52,55-59,443), it was hypothesized that the negative regulation of *APP* gene expression represents a defensive mechanism that could be achieved through increased expression of miR-101. To the best of our knowledge, the elevated miR-101 expression in AD patients has not been shown by

others. However, the research results on polymorphisms of *APP* gene and miRNAs deregulated in AD might be in accordance with this hypothesis (451,452). In a study on the cell culture model of AD that was conducted in 2021, an SNP-thiamine-cytosine substitution of the miR-101 premature form was detected and found to be related to the increased production of the mature, biologically active miR-101 molecule (452). The authors cautiously conclude that this variant is associated with the risk of AD, but do not provide potential explanations. Namely, looking from the pathophysiological point of view, this result may seem contradictory – miR-101 downregulates *APP* expression but the accelerated APP metabolism together with the *APP* overexpression is among the main pathological features of AD. Similarly, the AD-specific SNP of the *APP* 3'UTR-A454G has been demonstrated to enhance the effect of miR-20a, which, like miR-101, negatively regulates *APP* gene expression (451). Given that this finding implies decreased *APP* expression, the authors remain unclear and note that it deviates from the established concept of *APP* overexpression in AD. These results, however, may reflect complex events in this disease, which do not indicate a pathogenic process, but aim to protect the organism and oppose pathological circumstances, which is an essential principle of physiological and pathophysiological functions of the human organism.

Further considerations of this result, involved the fact that the study sample is heterogeneous based on the cognitive staging, with half of the examinees in the early AD. Therefore, a comparison of miR-101 expression levels between EAD and CAC groups showed significant up-regulation in this AD subgroup, relative to the CAC population. Interestingly, the results also indicated a decreasing trend of miR-101 expression with the progression of the disease towards the later stage, approaching the level of expression in the CAC group (Figures 18 and 19). Therefore, no significant difference was observed between AAD and CAC groups, implying that patients in the advanced disease stage did not contribute to the significant difference observed between AD and CAC groups (Figures 18 and 19). Therefore, after an attempt of the organism to counteract the accelerated APP metabolism to a toxic product, through the increase of miR-101 expression, its subsequent decline might be a new, logical consequence of the disease progression. Namely, as the production of Aβ increases, introducing neurons into the degenerative process, it can be assumed that increased and

accelerated APP metabolism, which potentially triggered miR-101 up-regulation, is now being reduced, leading to the subsequent decrease of miR-101 expression level. **So, it is possible that the overall miR-101 action could be understood as part of a negative feedback mechanism**. Actually, there are data that indicate that APP metabolism is not constant through the stages of AD. From the study by Holsinger et al., comes an important finding, that APP processing is increased specifically in early AD (458). Apart from that, there is a large body of evidence showing that the dynamics of APP processing are influenced by changes in neuron function. The status of synaptic activity can affect the metabolism of APP (459*)*, which is present in the presynaptic as well as in the postsynaptic compartment. In AD, synaptic transmission is significantly compromised by the neurodegenerative process (29,31,32,51,460). Therefore, it is possible, that with the progression of AD, the accumulation of Aβ and the consequent impairment of synaptic function, may be the reason for the reported changes in the dynamics of APP metabolism in the early compared to late disease stages (458). Thus, as the regulator of *APP* gene expression and APP metabolism, miR-101 initial up-regulation might be caused by accelerated APP metabolism, but, with its reduction in the later stages, miR-101 expression decreases through the negative feedback mechanism. An interesting study that explored the role of miR-384 in AD, which is a negative regulator of the *APP* gene, as well as miR-101, provides evidence to support the presented hypothetical explanation of our results. The authors first demonstrated a decrease of *APP* expression on cell culture caused by miR-384 and then reversibility of that effect using specific miR-384 inhibitor oligonucleotide (461). In contrast to a similar experiment with miR-101 (445) described earlier, a step further has been done in this research – treatment of the cell line with $A\beta_{42}$ continuously reduced miR-384 expression level, which confirmed the negative feedback mechanism between amyloid proteins and miR-384 (461). So far, a similar experimental setting has not been used for the investigation of miR-101 role in the regulation of *APP* expression.

When it comes to clinical evidence that might be relevant for consideration of the proposed mechanistic concept of miR-101 regulation in AD, there are some studies worth discussing. Namely, a negative correlation between plaque density in AD patients and miR-101 expression levels in CSF and serum has been found (462). An observation that the decrease

of miR-101 expression in CSF of AD patients correlates with the increment of plaque density has been understood as evidence of miR-101 disease-contributing effect. However, a view from another angle and the possibility that miR-101 might also gradually decrease as a consequence of decreased APP metabolism and Aβ accumulation should be strongly considered. Moreover, the causal context of miR-101 role, together with the fact that amyloid accumulates for a long period of the preclinical AD phase, rather implies that this miRNA should be already down-regulated at the moment of the disease diagnosis (or maybe even before the significant increase in amyloid load). It seems that this dynamic component of the negative correlation between plaque density and miR-101 expression fits better in the negative feedback hypothesis.

In order to further explore the dynamics and timing of miR-101 dysregulation in AD, literature data on its regulation in MCI has been investigated. However, there are very few studies concerning miR-101 and MCI. In support of the presented hypothesis, miR-101 was not deregulated in MCI patients who progressed to AD, in one study (351). In addition, the patients had noticeable amyloid pathology at the moment of circulatory miR-101 determination (351). This finding does not support the accepted considerations about the AD-contributing role of miR-101 that can be found in the literature. Actually, if it was causally associated with AD, it would rather be down-regulated at the moment of significant amyloid pathology. On the other side, indicated finding is more in accordance with our results of normal miR-101 expression levels and up-regulatory trend in early AD cases, as well as with our hypothesis, that the reason for its down-regulation in the advanced cases might be progressive amyloid pathology, all connected by the proposed negative feedback mechanism. Finally, miR-101 has been already described to act within the negative feedback mechanism in studies focused on cancer research. Jing et al. have described miR-101 involvement in the pathogenesis of CAC through the specific regulatory feedback circuit (463). Then, the results of the research on hepatocellular cancer, have revealed that the miR-101 oncosuppressive role has been inhibited through the other prooncogenic mechanism (464). This also supports the consideration that miR-101 dysregulation may act consequently, within the more complex setting of pathological events, rather than as the simple disease-contributing factor.

5.4.2. Circulatory miR–101 levels and their clinical implications

The result of the miR-101 expression level presented here provides potentially new insight into the possible mechanism of miR-101 dysregulation in AD but also its **implications for a potential clinical benefit.** Given that the variation of its expression may reflect dynamics of Aβ accumulation, it may not only indicate the disease progression, but more importantly, it could serve to monitor therapeutic effects. Amyloid-lowering therapy - **aducanumab and lecanemab,** are monoclonal antibodies recently approved by the FDA, indicated in the early AD population (202,465). Certainly, confirmation of amyloid pathology is a critical criterion for the selection of subjects for this treatment (68). When assessed with amyloid PET, amyloid pathology is not detectable in all the patients who are in early AD, according to a cognitive staging. In as many as 40% of them, this pathomorphological criterion is not met (466). Besides, due to the increased incidence of the adverse effects of the treatment, MRI is indicated before starting the therapy, during the dose titration, as well as in the case of certain symptoms or manifestations, reported by the patient (68). Presented results suggest that circulating miR-101 expression levels may have the potential to assist in the patient selection process for therapy, as well as in monitoring its effects. Actually, as already discussed, up-regulation of this miRNA detected in the early AD cases in the present study indicates that the normal or higher miR-101 expression level probably implies an early degree of amyloid pathology. On the other hand, its down-regulating trend in advanced cases might reflect a progressive amyloid load that leaves no room for the action of this therapy. Furthermore, in the context of monitoring the therapy effects, the miR-101 level of expression that is maintained stable would indicate a therapeutic benefit. Taking into account the purpose of miR-101 activity, which is a negative regulation of the *APP* gene (315,433,445-447), as well as the fact that progressive amyloid burden might cause a decrease in miR-101 expression the amyloid lowering therapy could also support natural defense mechanisms. Normal regulation of miR-101 would then contribute to the synergistic action and potentially improve the benefit from the therapeutic effect.

It is important to mention the study which conducted characterization of miR-101 expression in six different cell lines that ranged in their degree of differentiation from naïve to well differentiated. Although its expression levels were comparable among the tested cell lines,

the cells that represented CNS differentiated neurons demonstrated the highest expression of miR-101 (445). Guided by the results of this study, miR-101 expression levels in non-neuronal cells or circulation are expected to be lower. Therefore, the up-regulatory trend of circulatory miR-101 expression in the present study might be a reflection of the even more increased expression level of miR-101 in CNS neurons, which would support the hypothesis of miR-101 regulation, proposed in this research. In addition, some studies even demonstrated different miR-101 expression in the different brain cells (467).

In the end, there is a study that does not seem to support the proposed hypothesis of miR-101 acting within the negative feedback mechanism. This miRNA was found to be down-regulated in AD mouse models compared to the healthy, age-matched controls, regardless of the disease stage (449). Actually, in the AD model of the mouse, in which the pathology of the disease progresses with age, the miR-101 expression level was decreased in the hippocampus of young as well as old mice, compared to the controls (449). The reason for the discrepancy between these and the results of our study may lie in the use of a different model as well as a tissue type used to determine the level of miR-101.

A relatively small number of the subjects in the study presented here certainly represents a disadvantage. Therefore, in order to provide stronger evidence for indicated conclusions and proposed hypothesis of miR-101 regulatory action and potential application, research on a larger sample is warranted.

5.4.3. Cancer and Alzheimer's disease - hypothesis on a common miR-101- mediated regulation of signaling pathways

Research into the molecular relationship between AD and cancer has led us, as discussed, to interesting and potentially significant implications for AD. In addition, the inverse expression level of miR-101 in patients with AD and CAC was also considered in the context of the potential inverse molecular relationship between these two diseases. This miRNA was significantly up-regulated in AD compared to the CAC patients. So, we tried to understand whether miR-101 could represent a kind of overlapping point between AD and CAC and provide a molecular explanation for the inverse incidences of these two diseases.

Dysregulation of miRNAs is a highly represented pathogenic aspect in many malignancies (270,282,297,298,303-305,311-314,317,318,320,322-334). Change in the miR-101 expression level was first demonstrated on breast cancer cell lines, where it was found to be down-regulated (468). With the growing research interest in the role of this miRNA in cancer, its down-regulation was then proven in various other types of cancer (317,318,469,470,471). One of the earliest studies that explored the clinical relevance of miRNAs in CAC, reported that among others, miR-101 was barely detected in any tissue sample (472). Then, multiple studies have identified significant miR-101 down-regulation in CAC tissue and many types of colorectal cell lines (314,321,473,474). Namely, it has been shown that miR-101 has a defensive purpose, mediating tumor suppression (313,314,318,474-479). Its anti-tumor effects, inhibition of the development and progression of CAC through multiple targets, have been unambiguously demonstrated, in various experimental settings (314,321,474,477,478). Thus, decreased miR-101 expression in CAC has been associated with the reduced oncosuppression that it normally performs, and it is considered the CAC-contributing factor. Therefore, this literature data is in accordance with the result of miR-101 down-regulation in CAC patients, found in the present study.

Regarding the AD-CAC relationship, the evidence of the oncosuppressive miR-101 effect, together with the results of the present research, indicating an up-regulatory trend of miR-101 in the early AD, may lead to the following conclusion: long preclinical AD phase that can last for a couple of decades (29,30,44,45,105,106), might stimulate miR-101 up-regulation as a protective mechanism - to reduce *APP* gene expression, but also, during the same time, it enhances an oncosuppresive role of miR-101, potentially protecting these individuals from getting cancer. Actually, this hypothesis about the inverse coincidences of AD and CAC, emerging from our research, provides one of the possible explanations for why people who develop AD could have a lower risk of cancer.

However, whether such a conclusion is also true in the reverse case cannot be argued by the results of this study. In accordance with the stated hypothesis, an answer to that question could be potentially provided by the determination of the miR-101 expression level in the early CAC population. However, the presented study included only the CAC patients in the manifest, preoperative stage of the disease. Nevertheless, taking into account an

oncosuppressive role of miR-101, as well as the results from the present study on its action in AD, we can postulate the following hypothesis: miR-101 might be up-regulated in the early phase of CAC, to counteract the pro-oncogenic processes and simultaneously protect that population of patients from developing AD through the enhanced suppression of *APP*. Although the proposed hypothesis reflects an inverse relationship between these diseases, it does not necessarily imply the inverse expression values of miR-101, as found in this study. Instead, we propose that AD-triggered increased expression of miR-101 is also beneficial in protection from cancer. Due to the gradual decrease of miR-101 expression with the progression of AD, the significant difference between advanced AD cases and CAC patients who were also in the advanced, preoperative stage of the disease, was lost. Thus, we hypothesized that miR-101 expression has a down-regulating trend in the advanced stages of both diseases. This finding demonstrates that opposite miRNA values are not necessarily expected to explain the inverse relationship of these diseases, as already shown by others (295,296). In fact, the inverse expression values of this miRNA are likely caused by the heterogeneity of the AD group - which consisted of patients in the early and advanced disease stages, while in contrast to this, all the CAC patients were in an advanced, preoperative stage of the disease. Related to that, we assume that the miR-101 significant decrease in the patients with the advanced CAC detected by our and other studies might be the result of a negative feedback regulatory circuit, as we proposed for AD, and which has been already demonstrated for miR-101 in CAC and other cancer (463,464). An interesting study that explored alterations of miRNAs related to cigarette smoke in patients diagnosed with head and neck squamous cell carcinomas, detected down-regulation of miR-101 (320). However, exposure of normal epithelial cells to cigarette smoke-induced increased expression of miR-101, which implies that this miRNA must play an important role in the process of early carcinogenesis (320). Results of this study, which indicate early miR-101 up-regulation and its decreased expression in an invasive stage of cancer, support our hypothesis about miR-101 action within the negative feedback mechanism.

Simultaneous negative regulation of oncogenes and *APP* gene, through the up-regulation of miR-101, as the overlapping point of different signaling pathways, might explain the opposite incidences of AD and CAC. However, as a step further, we have tried to investigate whether

is there a common miR-101 target for both diseases, through which it would achieve supposed opposite effects, defending even more firmly its role as a molecule - an indicator of the inverse association of AD and CAC.

Studies related to miR-101 as a negative regulator of the cyclooxygenase - 2 (*COX - 2*) gene go in support of the presented hypothesis. *COX– 2* gene is implicated in many neoplastic and inflammatory disorders (317,318,480-482). Actually, changes in its expression levels and enzymatic activity are clearly related to AD, as well as to colorectal cancer pathology (480,483-485). MiR-101 inhibits the translation of *COX-2* mRNA, mediating tumor suppression that way (473,486). Similarly, suppression of *COX-2*, which is an important mediator of neuroinflammation, was demonstrated to be beneficial for the limitation of the damaging inflammatory processes characteristic of AD (487,488). Further on, it has been shown that miR-101 down-regulation was associated with *COX-2* overexpression in human colorectal cancer cells (473). Lack of *COX-2* inhibition due to miR-101 down-regulation in other cancers has been confirmed as well (318,489). Moreover, COX-2 inhibitors have been proposed as preventive CAC therapy (490), and particular drugs have been approved by the FDA as adjunction the usual care of patients with familial polypus adenomatosis to prevent its malignant transformation (491). Extensive research is conducted to investigate the potential benefit of COX-2 inhibitors in cancer treatment (490,492,493). Similarly, in AD, many efforts have been put into the research on blocking or deleting the *COX-2* gene, with the aim of development of therapeutic solutions (494,495,496). One of the interesting results and points, reached by thorough research on COX-2 activity in AD, indicates that it changes expression level with the progression of the disease - it is increased in the early stages but followed by the gradual decrement in advanced stages (497,498,499). In the present research, we detected an up-regulatory trend of miR-101 expression with a trend for a decrease in the advanced stages. Therefore, we assume that *COX-2* up-regulation in the early AD stage might cause miR-101 increased expression with the aim to suppress the *COX-2* gene and contribute to the limitation of the neuroinflammatory process. It has been already demonstrated that miR-101 overexpression decreased *COX-2* expression (317). Later on, in the chronic inflammatory phase, decreased *COX-2* level has been detected, so, through the negative feedback mechanism this might induce miR-101 down-regulation. As additional support to

such an action of miR-101 in the context of cancer, reduced miR-101 regulation was related to loss of *COX-2* inhibition but in a later stage of carcinogenesis (318). The latter fact, thus, might encourage a conclusion about miR-101 action within the negative feedback mechanism. Since its dysregulation happens after the many other events in the prooncogenic cascade, there is a high probability that it occurs consequently, rather than with the purpose of primary contribution to carcinogenesis, because, in that case, it would probably happen earlier in the disease process.

After these considerations, based on miR-101 action on the concrete target involved in both diseases, we reach the same conclusion - the nature of the AD-CAC relationship is inverse but not very likely reflected in the inverse miR-101 expression level. Instead, identical miR-101 regulation is rather beneficial for both diseases: an increase of COX-2 activity reported in early AD neuroinflammatory process might trigger miR-101 up-regulation found in our study, to suppress *COX-2* as a compensatory mechanism, which, at the same time inhibits oncogenic *COX-2* action. In other words, a change of miR-101 expression induced by AD is expected to protect these individuals from cancer through the identical regulatory effect on *COX-2*, the target shared by both diseases, which mediates neuroinflammation as well as carcinogenesis. That explains why people who have AD could have a reduced risk of cancer. In summary, based on the results of this study, and the fact that miR-101 is involved in the pathogenesis of both diseases - AD and CAC, we explored possibilities of its action that could explain inverse incidences of these diseases. Regardless of whether we discuss the effects of miR-101 simultaneous regulation on different targets or on a target common to both diseases, we come to the conclusion that the inverse relationship between these diseases is certain and reflected through altered miR-101 expression, induced by one disease. Therefore, the **results of the presented research confirm the third hypothesis.** Moreover, exploring the inverse relationship between AD and CAC, based on the results of this study, has helped us to discover potentially new explanations of miR-101 role in AD, that have not been demonstrated by others. Thus, **finally, this research also resulted in the establishment of a new scientific hypothesis.**

5.4.4. The potential impact of demographic and clinical variables on the miRNA expression level

In order to exclude the potential impact of demographic and clinical variables on the presented results, related to the miR-101 expression level in AD and CAC patients, additional statistical analyses were performed. Although CTRL, AD and CAC groups did not differ regarding the history of smoking (Table 9), when all participants were analyzed together, multiple regression analysis showed that smoking habits were negatively correlated with the miR-101 expression. Further analysis of each variable in a simple linear regression model confirmed that AD, CAC and history of smoking, individually had a significant influence on miR-101 expression level (Figure 20). CAC and smoking habits were associated with its down-regulation, and AD led to increased expression of miR-101. Thus, the results of the regression analysis were in agreement with the presented data on inverse miR-101 expression level between AD and CAC. In addition, the influence of cigarette consumption affected miR-101 expression in the same way as CAC suggesting their additive or synergistic effect. Dysregulation of miR-101 has already been associated with the impact of cigarette smoke in chronic obstructive pulmonary disease as well as in some cancers (317,320,500). An interesting study detected down-regulation of miR-101 related to cigarette smoke in patients diagnosed with head and neck squamous cell carcinomas (320). Other research found down-regulation of miR-101 in the human esophageal squamous cancer cell line - ECA109 as a response to cigarette smoke exposure (317). Therefore, to completely understand the results of miR-101 regulation among the study groups, it was important to analyze its expression level in each specific study group, taking into account the smoking habits of the examinees. In other words, it was necessary to clarify whether, in individuals suffering from AD or CAC, the impact of cigarette smoking is strong enough to influence the change of miR-101 expression differently, or more than the disease itself. Simple linear regression model analysis has shown that the presence of data related to the history of smoking of AD or CAC participants did not significantly contribute to the change of the miR-101 expression level (Figure 21). Therefore, it is possible that this study suggests a mechanism by which cigarette consumption, as a well-known, strong risk factor for CAC (501), contributes to the

development of CAC - through the down-regulation of miR-101, as has been shown in esophageal cancer (317).

In conclusion, demographic and clinical variables did not have a significant impact on presented differences in the expression level of miR-101 among the patient groups compared in this study. Therefore, the observed difference in the expression level of miR-101 between AD and CAC groups most probably emerges from the impact of the pathophysiological context of the particular disease.

6. CONCLUSIONS AND FUTURE DIRECTIONS:

Among the five investigated miRNA molecules, as many as three potential biomarkers of AD have been identified. Two of them could be of great importance for the stratification of the general population according to the risk of AD. Unexpectedly, the present study also identified one miRNA that could potentially serve for further stratification of patients with defined AD, or in the selection for the application of the new, disease-modifying therapy. As this miRNA might reflect the dynamics of the pathophysiological AD process targeted by this therapy, it could be potentially used also in the monitoring of the treatment efficacy. The simplicity, affordability, and non-invasiveness of the proposed method represent the additional value and strengthen the possibility of future application of these miRNAs in the AD field. Apart from this application in the AD context, the results of the presented research offer molecular confirmation of the AD-CAC inverse relationship.

More specifically, the conclusions of the present research would be:

1. This study accidentally identified that a certain number of patients with cognitive decline in the Montenegrin population remain undetected. SCD evaluation should be an important and possibly critical aspect of the successful and timely detection of cognitive decline, but neuropsychological screening instruments should be routinely administered to the elderly in Montenegro, even if the patient does not complain of problems related to cognitive functioning.
2. The upregulation of miR-146a and miR-155 could serve as a non-invasive, circulatory biomarker for the detection of people with cognitive decline who are at risk for

AD and thus, potentially, also for monitoring of drug treatment efficacy and for making the prognosis for patients in early stages of AD.

3. Neuropsychological screening instruments and molecular markers identified by this study could be used together to significantly improve our ability to diagnose AD in the very early stage and could possibly become routine non-invasive tools for screening the general population for AD.

4. The up-regulating trend of miR-101 in early AD and its decline with disease progression found in the presented study, suggests that miR-101 might act within the negative feedback mechanism related to APP metabolism. This has not been demonstrated by others and represents a new scientific hypothesis.

5. Considering miR-101's oncosuppressive role, increased miR-101 expression in long-lasting preclinical and early AD might protect AD patients from cancer. In fact, simultaneous negative regulation of oncogenes and *APP* gene, through the up-regulation of miR-101, as the overlapping point of different signaling pathways, might explain the opposite incidences of AD and CAC.

The number of participants is the limiting aspect of our study, thus, research on the larger group is warranted in the future. Moreover, the lack of a complete neurological evaluation in CTRL and LP-MoCA groups, which includes invasive and expensive tests, might represent another limiting point of our study, but at the same time, it has opened new directions for future cohort studies with the LP-MoCA group. Regular follow-ups, and screening of molecular and clinical inflammatory markers with complete neurological assessment at later time points will potentially confirm AD as a cause of their cognitive impairment, and emphasize the potential significance of inflamma-miR-146a and -155 in the early detection of AD. Although the determination of inflamma-miR-146a and -155 circulatory levels might represent a novel non-invasive biomarker for the detection of an early stage of cognitive impairment due to AD, additionally altered miRNA and/or small non-coding RNA levels may be uncovered and further improve the use of non-invasive, circulatory biomarkers for the diagnosis, drug treatment efficacy monitoring and prognosis of early AD stages. Also,

the cross-cultural validity of the MoCA cutoff score and its adjustment for our region should be explored in the future. Eventually, the results of our study imply that SCD evaluation through an open question might not always be a reliable tool to indicate CI in the elderly. Therefore, the proposed structured evaluation form (155) should be considered as an assessment tool in future studies.

MiR-101 potentially reflects a progression of amyloid accumulation, so, as opposed to currently indicated expensive and hardly accessible methods, future investigation of its potential to serve in the patient selection process as well as in monitoring the effects of amyloid lowering therapy might be of great importance. As a step further in the potential confirmation of this hypothesis, a study on a larger sample that would compare miR-101 expression with the findings on amyloid PET imaging would be needed.

Finally, a realization of this study, together with the results indicated, has led to the conclusion that such a complex disease as AD cannot be detected only by one particular biomarker. It is more likely that from challenging molecular interactions in the pathogenetic course of AD, additional important markers will emerge, some of them more specific for the detection of AD in the early stages, while others will be more specific for the prediction of disease progression and some will be a therapeutic solution.

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8. ATTACHMENTS:

Attachment 1.

INFORMATION FOR THE PATIENTS WITH AD

Dear (Name) _____(Surname)_____

You are invited to participate in the research which is conducted in the Clinical Center of Montenegro and at the Faculty of Medicine.

Your participation in the research is anonymous and voluntarily.

If you decide that you do not want to participate in the research, you are able to give up any moment, without the obligation to explain your decision.

All the information obtained through this research will be strictly confidential, in accordance with the Law on the Taking and Use of Biological Samples (Sve informacije dobijene ovim istraživanjima biće strogo čuvane i povjerljive, u skladu sa Zakonom o uzimanju i korištenju bioloških uzoraka (Official Gazette of Montenegro No. 14/2010 since 03.17.2010.) and the Law on Protection of Genetic Data (Official Gazette of Montenegro No. 25/2010).

The aim of the research

To improve the risk assessment for progression of the disease and its complications in the older age (e.g. Dementia), using the new methods. This would help to determine in a much

more precise way the most effective therapy and thus increases the chances to improve quality of life, survival, reducing of the complications and, in some cases, healing.

Methods of the research

Collection of the information and biological samples will be conducted once or up to 2 times in a year.

- Questionnaire and the physical examination with the short examination of the mental state/mental state check. These methods will help us to understand better your health and lifestyle habits.

- Retinal/fundus examination. This is part of the usual ophathalmology examination. The doctor will take the simple photography of the left and right eyes. He will use camera with the special lanses which allowes us to examine fundus/ bottom of the eye.

- Collecting of the vein blood sample.

- 10 ml of vein blood will be taken for the scientific research.

- In case that during this study, which lasts until April 2020, you get an infection that requires a hospital treatment, two more samples of vein blood will be collected. These additional samples would be the part of the standard hospital diagnostic and therapeutic protocols, and a small part would be used for the scientific research.

WRITTEN INFORMED CONSENT FOR THE PARTICIPATION IN THE STUDY

I confirm that I have read and understood purpose of the research and planned research methods and I agree to participate in the research.

Signature of the examinee or his legal representative_____

Signature of the researcher_____

Date_____

INFORMATION FOR THE PATIENT with CAC

Dear (Name) _____(Surname)_____

You are invited to participate in the research which is conducted in the Clinical Center of Montenegro and at the Faculty of Medicine.

Your participation in the research is anonymous and voluntarily.

If you decide that you do not want to participate in the research, you are able to give up any moment, without the obligation to explain your decision.

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To improve risk assessment for progression of the disease and its complications in the older age (e.g. Dementia), using the new methods. This would help to determine in a much more precise way the most effective therapy and thus increases the chances to improve quality of life, survival, reducing of the complications and, in some cases, healing.

Methods of the research

Collection of the information and biological samples will be conducted once or up to 2 times in a year.

- Questionnaire and the physical examination with the short examination of the mental state/mental state check. These methods will help us to understand better your health and lifestyle habits.

- Retinal/fundus examination. This is part of the usual ophathalmology examination. The doctor will take the simple photography of the left and right eyes. He will use camera with the special lanses which allowes us to examine fundus/ bottom of the eye.

- Collecting of the vein blood sample.

- 10 ml of vein blood will be taken for the scientific research.

- In case that during this study, which lasts until April 2020, you get an infection that requires a hospital treatment, two more samples of vein blood will be collected. These additional samples would be the part of the standard hospital diagnostic and therapeutic protocols, and a small part would be used for the scientific research.

IN EXAMINEES WHO NEED SURGICAL TREATMENT:

- Tissue sampling. In patients diagnosed with colorectal cancer, tumor tissue usually has to be surgically removed for the purpose of treatment. Qualyfied physisians at the Center for Pathology in CCM, always analyze removed tumor tissue, in order to obtain precise data about the tumor, which are important for the efficient and better treatment. From the rest of the tissue, which was not used for this standard diagnostic procedure, and which otherwise would be disposed as medical waste, the doctor will take a small part for additional histological and molecular analyses in the Center for scientific research of Faculty of Medicine in Podgorica.

- Collection of the vein blood. In the postoperative period, 10 ml of vein blood will be collected 24 and 48 hours after the surgery, what is also part of the standard postoperative protocol, and one part of that blood sample will be used for the scientific research.

WRITTEN INFORMED CONSENT FOR THE PARTICIPATION IN THE STUDY

I confirm that I have read and understood purpose of the research and planned research methods and I agree to participate in the research.

Signature of the examinee or his legal representative_____

Signature of the researcher_____

Date_____

Attachment 2.

QUESTIONNAIRE

Date_____

The questionnaire is conducted by_____

Demographic data	
Serial number	
Date of birth	
Age at the moment of examination	
Gender (round reply)	male female
Body weight	
Body height	
Level of education	write in the number of years of education

List of medications you take regularly

Personal health data	
Have you ever had to spend the night in the hospital?	YES NO
Why?	
Have you ever had surgery?	YES NO
Why?	
Has the doctor ever suggested you a heart test (e.g. ECG, stress test, echocardiography), or? any other diagnostic procedure (colonoscopy, mammography or something like that)?	YES NO
Which test/procedure?	

Personal health data	
Are you being treated or have been treated in the past because of?	
High blood pressure	YES NO
High level of cholesterol or triglycerides	YES NO
Diabetes	YES NO
Heart attack	YES NO
Stroke	YES NO
Heart failure (chronic cardiac insufficiency)	YES NO
Circulation problems (intermittent claudication)	YES NO
Malignant tumor	YES NO
If the answer to this question is YES, specify which tumor	
Have you ever been treated with chemotherapy?	YES NO
Have you ever been treated with radio therapy?	YES NO
Have you ever had any neurological disease?	YES NO
If the answer to this question is YES, specify which one	
Have you been diagnosed with Alzheimer's disease in the past? If the answer to this question is YES, answer the following 2 questions below	YES NO
How old were you at the beginning of this disease	
How long has it been since the onset of the disease, until the diagnosis?	

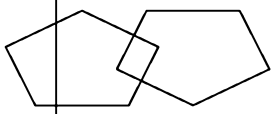
Attachment 3. Neuropsychological screening tests

Mini-Mental State Examination (MMSE)

Patient's Name: _____ Date: _____

Instructions: ***Ask the questions in the order listed. Score one point for each correct*** **response within each question or activity.**

Maximum Score	Patient's Score	Questions
5		"What is the year? Season? Date? Day of the week? Month?"
5		"Where are we now: State? County? Town/city? Hospital? Floor?"
3		The examiner names three unrelated objects clearly and slowly, then asks the patient to name all three of them. The patient's response is used for scoring. The examiner repeats them until patient learns all of them, if possible. Number of trials: _____
5		"I would like you to count backward from 100 by sevens." (93, 86, 79, 72, 65, …) Stop after five answers. Alternative: "Spell WORLD backwards." (D-L-R-O-W)
3		"Earlier I told you the names of three things. Can you tell me what those were?"
2		Show the patient two simple objects, such as a wristwatch and a pencil, and ask the patient to name them.
1		"Repeat the phrase: 'No ifs, ands, or buts.'"
3		"Take the paper in your right hand, fold it in half, and put it on the floor." (The examiner gives the patient a piece of blank paper.)
1		"Please read this and do what it says." (Written instruction is "Close your eyes.")

1		“Make up and write a sentence about anything.” (This sentence must contain a noun and a verb.)
1		“Please copy this picture.” (The examiner gives the patient a blank piece of paper and asks him/her to draw the symbol below. All 10 angles must be present and two must intersect.) 
30		TOTAL

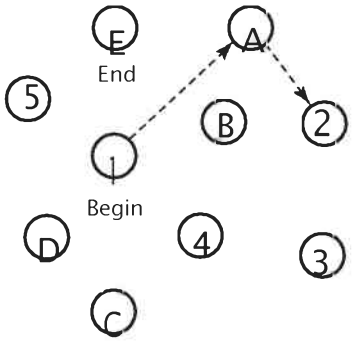
(Adapted from Rovner & Folstein, 1987)

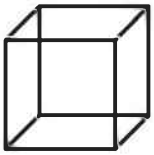
MONTREAL COGNITIVE ASSESSMENT (MOCA)

NAME :
Education :
Sex :

Date of birth :
DATE :

VISUOSPATIAL / EXECUTIVE





Copy cube

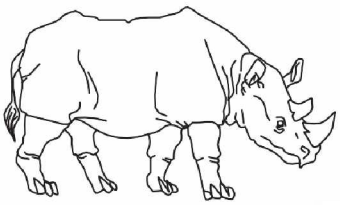
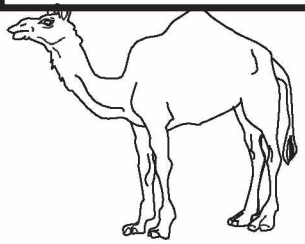
[ ] [ ]

Draw CLOCK (Ten past eleven)
(3 points)

[ ] Contour [ ] Numbers [ ] Hands

/5

NAMING

[ ] [ ] [ ]

/3

MEMORY

Read list of words, subject must repeat them. Do 2 trials. Do a recall after 5 minutes.

	FACE	VELVET	CHURCH	DAISY	RED
1st trial					
2nd trial					

No points

ATTENTION

Read list of digits (1 digit/ sec.). Subject has to repeat them in the forward order [ ] 2 1 8 5 4

Subject has to repeat them in the backward order [ ] 7 4 2

/2

Read list of letters. The subject must tap with his hand at each letter A. No points if ≥ 2 errors

[ ] F B A C M N A A J K L B A F A K D E A A A J A M O F A A B

/1

Serial 7 subtraction starting at 100 [ ] 93 [ ] 86 [ ] 79 [ ] 72 [ ] 65

4 or 5 correct subtractions: **3 pts**, 2 or 3 correct: **2 pts**, 1 correct: **1 pt**, 0 correct: **0 pt**

/3

LANGUAGE

Repeat : I only know that John is the one to help today. [ ]

The cat always hid under the couch when dogs were in the room. [ ]

/2

Fluency / Name maximum number of words in one minute that begin with the letter F [ ]_____(N ≥ 11 words)

/1

ABSTRACTION

Similarity between e.g. banana - orange = fruit [ ] train - bicycle [ ] watch - ruler

/2

DELAYED RECALL

	Has to recall words WITH NO CUE	FACE	VELVET	CHURCH	DAISY	RED	Points for UNCUED recall only
		[ ]	[ ]	[ ]	[ ]	[ ]	
Optional	Category cue						
	Multiple choice cue						

/5

ORIENTATION

[ ] Date [ ] Month [ ] Year [ ] Day [ ] Place [ ] City

/6

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Normal ≥ 26 / 30

Attachment 4.

Geriatric Depression Scale (Short Form)

Patient's Name:_____ Date:

_____

Instructions: Choose the best answer for how you felt over the past week. Note: when asking thepatient to complete the form, provide the self-rated form (included on the following page).

No.	Question	Answer	Score
1.	Are you basically satisfied with your life?	YES / ***NO***	
2.	Have you dropped many of your activities and interests?	***YES*** / NO	
3.	Do you feel that your life is empty?	***YES*** / NO	
4.	Do you often get bored?	***YES*** / NO	
5.	Are you in good spirits most of the time?	YES / ***NO***	
6.	Are you afraid that something bad is going to happen to you?	***YES*** / NO	
7.	Do you feel happy most of the time?	YES / ***NO***	
8.	Do you often feel helpless?	***YES*** / NO	
9.	Do you prefer to stay at home, rather than going out and doing new things?	***YES*** / NO	
10.	Do you feel you have more problems with memory than most people?	***YES*** / NO	
11.	Do you think it is wonderful to be alive?	YES / ***NO***	
12.	Do you feel pretty worthless the way you are now?	***YES*** / NO	
13.	Do you feel full of energy?	YES / ***NO***	
14.	Do you feel that your situation is hopeless?	***YES*** / NO	
15.	Do you think that most people are better off than you are?	***YES*** / NO	
		TOTAL	

(Sheikh & Yesavage, 1986)

Attachment 5. Target genes of miR - 146a

Breast cancer type 1 susceptibility protein; **BRCA1**	Cyclooxigenase-2; **COX2**	Interleukin 1 receptor like 2; **IL1RL2**
Breast cancer type 1 susceptibility protein; **BRCA2**	Myeloid differentiation primary response protein MyD88; **MYD88**[c]	Metastsis-associated gene family, member 2; **MTA2**
Caspase-7; **CASP7** [a]	Unconventional myosin-VI; **MYO6**	Adiponectin, C1Q and collagen domain containing; **ADIPOQ**
Cyclin-A2; **CCNA2**	Nuclear factor NF-kappa-B p105 subunit; **NFKB1**[a,b]	BCL2 associated transcription factor 1; **BCLAF1**
CD40 ligand; **CD40LG**	Neurogenic locus notch homolog protein 2; **NOTCH2**	Wiskott-Aldrich syndrome protein family member 2; **WASF2**
Cyclin-dependent kinase inhibitor 1; **CDKN1A**	Proliferation-associated protein 2G4; **PA2G4**	COP9 signalosome subunit 9; **COPS8**
Cyclin-dependent kinase inhibitor 3; **CDKN3**	Protein Kinase C epsilon; **PRKCE**	FAS-associated factor 1; **FAF1**
Carboxypeptidase M; **CPM**	Prostaglandin-endoperoxide synthase 2; **PTGS2**[a,b]	Erb-B2 receptor tyrosine kinase 4; **ERBB4**
Cellular communication network factor 2; **CTGF**	RAS-related C3 botulinum toxin substrate; **RAC1**[c]	TGF-beta activated kinase 1(MAP3K7) binding protein; **TAB2**

Dual specificity protein phosphatase 1; **DUSP1**	Retinoic acid receptor beta; **RARB**	Ring finger protein 11; **RNF11**
Epidermal growth factor receptor; **EGFR**[c]	Roundabout homolog 1; **ROBO1**	Glutaminase 2; **GLS2**
ELAV-like protein 1; **ELAVL1**	Rho associated coiled-coil containing protein kinase 1; **ROCK1**	Ubiquitin like with PHD and ring finger domains 1; **UHRF1**
Receptor tyrosine-protein kinase erbB-4; **ERBB4**	S100 calcium-binding protein A12; **S100A12**	Caspase recruitment domain family member 10; **CARD10**
Glutaminase kidney isoform, mitochondrial; **GLS**	Chemokine (C-C motif) ligand 5; **CCL5**	Interleukin-1 receptor-associated kinase 4; **IRAK4**
Complement factor H; **CFH**[c]	C-X-C Motif Chemokine Ligand 12; **CXCL12**	Zinc finger protein 117; **ZNF117**
Heterogeneous nuclear ribonucleoprotein a/b/d; **HNRNPD**	Secretory leukocyte peptidase inhibitor; **SLPI**	TP53 induced glycolysis regulatory phosphatase; **TIGAR**
Homeobox protein Hox-D10; **HOXD10**	Survival of motor neuron 1; **SMN1**	Prostaglandin E synthase 2; **PTGES2**[c]
Intercellular adhesion molecule 1; **ICAM1**[c]	Ras/Rac guanine nucleotide exchange factor 1; **SOS1**	Alt inducible kinase 1; **SIKE1**

Interleukin1 receptor accessory protein; **IL1RAP**	SRY-box transcription factor 2; **SOX2**	Rhophilin Rho GTPase binding protein 2; **RHPN2**
Interleukin-6; **IL6**[a,b]	Secreted Phosphoprotein 1; **SPP1**	CCR4-NOT transcription complex subunit 6 like; **CNOT6L**
Interleukin-8; **IL8**[c]	Signal transducer and activator of transcription1; **STAT1**	Metastasis associated lung adenocarcinoma transcript 1; **MALAT1**
Interleukin-1 receptor--associated kinase 1; **IRAK1**	Transcription factor Dp-2; **TFDP2**	Aquaporin 4; **AQP4**
Interleukin-1 receptor-associated kinase 2; **IRAK2**[c]	Toll-like receptor 2; **TLR2**	Calcium/calmodulin dependent protein kinase II alpha; **CAMK2A**
Kinesin-like protein KIF22; **KIF22**	Toll-like receptor 4; **TLR4**	C-reactive protein; **CRP**[c]
Neural cell adhesion molecule L1; **L1CAM**	TNF receptor associated factor 6; **TRAF6**	Desmoglein 2; **DSG2**
Laminin subunit gamma-2; **LAMC2**	C-X-C chemokine receptor type 4; **CXCR4**	Receptor tyrosine-protein kinase erbB-2; **ERBB2**
Tumor necrosis factor receptor superfamily member 6; **FAS****	Coiled-coil domain containing 6; **CCDC6**	GA binding protein transcription factor subunit alpha; **GABPA**

Interleukin 10; **IL10**	ST8 Alpha-N-acetyl-Neuraminide Alpha-2,8-Sialytransferase 4; **ST8SIA4**	Mothers against decapentaplegic homolog 2; **SMAD2**[c]
MAF BZIP transcription factor G; **MAFG**	Neuropilin 2; **NRP2**	Mothers against decapentaplegic homolog 4; **SMAD4**[c]
CEA cell adhesion molecule 6; **CEACAM6**	Pituitary tumor transforming gene 1; **PTTG1**	NUMB endocytic adaptor protein; **NUMB**
Nitric oxide synthase 1; **NOS1**[a]	Breast cancer metastasis suppressor 1; **BRMS1**	Bone gamma-carboxyglutamate protein; **BGLAP**
Phosphatase and tensin homolog; **PTEN**[c]	Hepatitis A virus cellular receptor 1 homolog; **HAVCR1**	Fas-associated via death domain; **FADD**[a,b,c]
Superoxide dismutase 2; **SOD2**	Reticulon 4; **RTN4**[a]	Glial fibrillary acidic protein; **GFAP**
Tumor-necrosis factor; **TNF**[a,b]		

[a] genes involved in pathogenesis of Alzheimer's disease; [b] AD - related genes with the role in immune and inflammatory response; [c] common target genes of miR - 146a and miR - 155.

Attachment 6. Target genes of miR – 155

Annexin A2; **ANXA2**	Meprin A subunit alpha; **MEP1A**	Zinc finger protein 28; **ZNF28**
Angiotensin II receptor type 1; **AGTR1**	Microphthalmia-associated transcription factor; **MITF**	Phosphatidylinositol-binding clathrin assembly protein; **PICALM**[a]
Apoptotic peptidase activating factor 1; **APAF1**	MutL homolog 1; **MLH1**	Suppressor of cytokine signaling 1; **SOCS1**
Adenomatous polyposis coli; **APC**	Mitogen-activated protein kinase 10; **MAP3K10**	Proliferation and apoptosis adaptor Protein 15; **PEA15**
Ras homolog family member A; **RHOA**	Matrix metallopeptidase 16; **MMP16**	FAS associated via death domain; **FADD**[a,b,c]
BTB and CNC homology 1; **BACH1**	MutS homolog 2; **MSH2**	B-cell lymphoma/leukemia 10; **BCL10**
Cyclin D1; **CCND1**	MAX interactor 1; **MXI1**	Mitogen-activated protein kinase 14; **MAP3K14**
B-cell lymphoma 2; **BCL2**	Transcriptional activator Myb; **MYB**	Suppressor of cytokine signaling 3; **SOCS3**
B-cell lymphoma 6; **BCL6***	MYC proto-oncogene; **MYC**	Claudin 1; **CLDN1**
Brain derived neurotrophic factor; **BDNF**[a]	Myb proto-oncogene like 1; **MYBL1***	Mfs transporter, pat family, solute carrier family 33 (Acetyl-CoA transporter), member 1; **SLC33A1 **[a]

Runt-related transcription factor 2; **RUNX2**	Myeloid differentiation primary response 88; **MYD88**[c]	Thyroid hormone receptor interactor 13; **TRIP13**
T-cell surface glycoprotein CD4; **CD4****[a,b]	Myosin light chain kinase; **MYLK**	Zinc finger protein 254; **ZNF254**
Cluster of differentiation 68; **CD68**	Myosin ID; **MYO1D**	Inhibitor of nuclear factor kappa B kinase subunit epsilon; **IKBKE**
Interleukin 1 receptor associated kinase 3; **IRAK3**	MYO10 myosin X; **MYO10**	SH3 and PX domain-containing protein 2A; **SH3PXD2A**
Mitogen-activated protein kinase 14; **MAPK14**	Asparaginyl-tRNA synthetase; **NARS**	Meiosis regulator and mRNA stability factor 1; **KIAA0430**
Macrophage colony-stimulating factor 1 receptor; **CSF1R****[a,b]	Nuclear factor erythroid 2--related factor 2; **NFE2L2**	PHD finger protein 14; **PHF14**
Dedicator of Cytokinesis; **DOCK1**	Nuclear factor kappa B Subunit 1; **NFKB1**	Rap guanine nucleotide exchange factor 2; **RAPGEF2***
Cut-like homeobox 1; **CUX1**	Homeobox protein Nkx-3.1; **NKX3-1**	Matrin-3; **MATR3****
Casein kinase 1 alpha 1; **CSNK1A1**	Nitric oxide synthase 2; **NOS2**	Cytoskeleton-associated protein 5; **CKAP5**
E2F transcription factor 2; **E2F2**	Nitric oxide synthase 3; **NOS3***	Translocase of outer mitochondrial membrane 20; **TOMM20**

Endothelin-1; **EDN1***	Neurogenic locus notch homolog protein 1; **NOTCH1***	MAF Bzip transcription factor B; **MAFB**
Polyhomeotic homolog 2; **PHC2**	NOVA Alternative S plicing Regulator 1; **NOVA1**	Actin-related protein 2/3 complex subunit 3; **ARPC3**
Epidermal growth factor receptor; **EGFR*[c]**	Oxidized low-density lipo-protein receptor 1; **OLR1**	Actin related protein 2; **ACTR2**
ETS proto-oncogene 1, tran-scription factor; **ETS1**	Phosphatidylethanolamine binding protein 1; **PEBP1**	Abl-interactor 2; **ABI2**
Fibroblast growth factor 7; **FGF7**	PAK2 p21 (RAC1) activated kinase 2; **PAK2**	Patj crumbs cell polarity complex component; InaD-like protein; **INADL**
Forkhead box O3; **FOXO3**	Protocadherin 9; **PCDH9**	Zinc finger and BTB domain con-taining 18; **ZBTB18**
Friend leukemia integration 1 transcription factor; **FLI1**	Phosphoinositide-3-kinase regulatory subunit 1; **PIK3R1**	Adp ribosylation factor like gtpase 6 interacting protein 5; **ARL6IP5**
Vascular endothelial growth factor receptor 1; **FLT1**	Plastin 1; **PLS1**	PDZ And LIM Domain 5; **PDLIM5**
Glycine amidinotransferase; **GATM**	Periplakin; **PPL**	PC4 And SRSF1 interacting Protein 1; **PSIP1**

Neuronal membrane glyco-protein M6-b; **GPM6B**	Camp-dependent protein ki-nase inhibitor alpha; **PKIA**	Aryl hydrocarbon receptor nuclear translocator-like protein 1; **ARNTL**
MutS homolog 6; **MSH6**	Trafficking kinesin-binding protein 1; **TRAK1***	CCAAT enhancer binding protein beta; **CEBPB**
Histidine ammonia-lyase; **HAL**	Protein kinase N2; **PKN2**	SRY-box transcription Factor 6; **SOX6**
Hypoxia-Inducible Factor 1alpha; **HIF1A**	Twinfilin actin binding protein 1; **TWF1**	Cut-like homeobox 1; **CUX1**
HIVEP zinc finger 2; **HIVEP2**	Pleiotrophin; **PTN**	Forkhead box protein P3; **FOXP3***
Intercellular adhesion molecule; **ICAM1**[c]	Ras-related C3 botulinum toxin substrate 1; **RAC1**[c]	Inositol polyphosphate-5--phosphatase F; **INPP5F**
Truncated interferon--gamma receptor 1; **IFNGR1**	DNA repair protein RAD51 homolog 1; **RAD51***	Jumonji and AT-rich interaction domain containing 2; **JARID2**
Cysteine-rich angiogenic inducer 61; **CYR61**	RHEB pseudogene 1; **RHEB**	Spi-1 proto-oncogene; **SFPI1**
Immunoglobulin J polypep-tide; **IGJ**	Syndecan binding protein; **SDCBP**	Src-like adaptor; **SLA**
Interleukin 1B; **IL1B***[a,b]	Protein sel-1 homolog 1; **SEL1L**	Zinc finger protein 652; **ZNF652**

Interleukin 2; **IL2**	E-selectin; **SELE**	Rab11 family-interacting protein2; **RAB11FIP2**
Interleukin 8; **IL8**[c]	Ski oncogene; **SKI**	Phosphatase and tensin homolog; **PTEN***
TAF5-like RNA polymerase II p300/CBP-associated factor-associated factor 65 kDa subunit 5L; **TAF5L**	Swi/snf related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4; **SMARCA4***	PRKAR1A protein kinase Camp-dependent type I regulatory subunit alpha; **PRKAR1A**
C-X-C motif chemokine ligand 10; **CXCL10**	Transcription factor SOX-1; **SOX1**	SH3 and multiple ankyrin repeat domains protein 2; **SHANK2**
Inositol polyphosphate--5-phosphatase D; **INPP5D**	Spi-1 Proto-Oncogene; **SPI1**	Tripartite Motif Containing 32; **TRIM32**
Interleukin 1 receptor associated kinase 2; **IRAK2**[c]	Tubulin-specific chaperone A; **TBCA**	Histone h3-n6,n6-dimethyl-l-lysine4 fad-dependent demethylase; **KDM1A**
Interleukin 2 inducible T cell kinase**;** **ITK**	GC-rich sequence DNA--binding factor 2; **GCFC2**	Clusterin-associated protein 1; **CLUAP1**
Jumonji and AT-rich interaction domain containing 2; **JARID2**	Transcription factor 12; **TCF12**	28S ribosomal protein S27, mitochondrial; **MRPS27**
Jun proto-oncogene, AP-1 transcription factor subunit; **JUN**	Telomeric repeat-binding factor 1; **TERF1**	TGF-beta-activated kinase 1 and MAP3K7-binding protein 2; **TAB2**

Kinase insert domain Receptor; **KDR**	Thyroid hormone receptor beta; **THRB**	WW and C2 domain containing1; **WWC1**
Kirsten rat sarcoma viral oncogene homolog; **KRAS**	Transducin-like enhancer protein 4; **TLE4**	Exosome complex component RRP4; **EXOSC2****
Mothers against decapentaplegic homolog 1; **SMAD1**	Zinc finger transcription factor Trps1; **TRPS1**	MORC family CW-type zinc finger protein 3; **MORC3**
Mothers against decapentaplegic homolog 2; **SMAD2[c]**	Transcription termination factor 1; **TTF1**	Calcium-regulated heat-stable protein 1; **CARHSP1**
Mothers against decapentaplegic homolog 3; **SMAD3**	Ubiquinol-cytochrome c reductase, rieske iron-sulfur polypeptide 1; **UQCRFS1**	LDOC1, regulator of NFKB signaling; **LDOC1**
Mothers against decapentaplegic homolog 4; **SMAD4[c]**	Vascular cell adhesion protein 1; **VCAM1**	Gamma-aminobutyric acid receptor-associated protein-like 1; **GABARAPL1**
Mothers against decapentaplegic homolog 5; **SMAD5**	Von Hippel-Lindau disease tumor suppressor; **VHL**	Kelch repeat and btb domain--containing protein 2; **KBTBD2**
Methyl-CpG binding protein 2; **MECP2***	Wee1-like protein kinase; **WEE1***	Regulator of nonsense transcripts 2; **UPF2**

Myocyte enhancer factor 2A; **MEF2A**	14-3-3 protein zeta/delta; **YWHAZ**[a]	Hect and rld domain containing e3 ubiquitin protein ligase 4; **HERC4**
Homeobox protein meis1; **MEIS1***	Zinc finger protein ZIC 3; **ZIC3**	Integrator complex subunit 6; **INTS6**
HMG box-containing protein 1; **HBP1**	Muscle blind-like protein 3; **MBNL3**	Cytochrome P450 2U1; **CYP2U1***
Interleukin 13 receptor subunit alpha 1; **IL13RA1**	Histone h3-dimethyl--l-lysine9 demethylase; **KDM3A**	Afadin-and alpha--actinin-binding protein; **SSX2IP**
Phosphatase domain containing, paladin 1; **PALD1**[a]	Cytokine induced apoptosis inhibitor 1; **CIAPIN1**	Anaphase-promoting complex subunit 16; **ANAPC16**
Bromodomain and PHD finger-containing protein 3; **BRPF3**	VPS35 endosomal protein sorting factor like; **C16ORF62**	RNA-binding protein Musashi homolog 2; **MSI2**
Arfaptin-1; **ARFIP1**	Zinc finger protein 248; **ZNF248**	Mitochondrial import inner membrane translocase subunit TIM14; **DNAJC19**
39S ribosomal protein L18, mitochondrial; **MRPL18**	TBC1 domain family member 14; **TBC1D14**	Family with sequence similarity 199, X-linked; **FAM199X**
Vesicle transport protein GOT1B; **GOLT1B**	Family with sequence similarity 135 member A; **FAM135A**	Zinc finger protein 714; **ZNF714**

Ubiquilin-1; **UBQLN1**	Teashirt homolog 3; **TSHZ3**	Protein FAM91A1; **FAM91A1**
Kelch-like protein 5; **KLHL5**	Sirtuin 1; **SIRT1**	Zinc finger protein 431; **ZNF431**
Coiled-coil domain containing 41; **CCDC41**	RB-associated KRAB zinc finger protein; **RBAK**	Eukaryotic translation initiation factor 2c; **AGO4**
Chromosome 3 open reading frame 18; **C3ORF18**	E3 ubiquitin-protein ligase RNF123; **RNF123**	Toll like receptor 3; **TLR3**
Lysine-rich coiled--coil protein 1; **KRCC1**	MAGUK p55 subfamily member 5; **MPP5**	Ligand of numb-protein X 2; **LNX2**
Pre-mRNA-processing factor 17; **CDC40**	Serine/threonine-protein kinase WNK1; **WNK1***	Programmed cell death protein 4; **PDCD4**
Calcium-binding protein 39; **CAB39**	Selenocysteine insertion sequence-binding protein 2; **SECISBP2**	Ligand-dependent nuclear receptor corepressor-like protein; **LCORL**
Transmembrane 6 super--family member 1; **TM6SF1**	Probable ATP-dependent RNA helicase DHX40; **DHX40**	Germinal center-associated signaling and motility protein; **GCSAM**
DNA polymerase epsilon 3, accessory subunit; **POLE3**	Histone deacetylase complex subunit SAP30L; **SAP30L***	Family with sequence similarity 177 member A1; **FAM177A1**

ADP-ribosylation factor--like protein 15; **ARL15**	Coiled-coil domain--containing protein 82; **CCDC82**	Zinc finger protein 493; **ZNF493**
Solute carrier family 35, member f1/2; **SLC35F2**	PHD finger protein 17; **PHF17**	Tumor protein p53-inducible nuclear protein 1; **TP53INP1**
WW domain binding protein 1 like; **WBP1L**	Chromodomain-helicase-DNA-binding protein 9; **CHD9**	Occludin; **OCLN***
TBC1 domain family member 8B; **TBC1D8B**	Tetraspanin-14; **TSPAN14**	Type-1 angiotensin II receptor; **AGTR1**
Wolf-Hirschhorn syndrome candidate 1-like protein 1; **WHSC1L1**	Zinc finger protein 611; **ZNF611**	NACHT, LRR and PYD domains-containing protein 3; **NLRP3***
Zinc finger protein ZIC 3; **C17ORF80**	Ras association domain-containing protein 4; **RASSF4**	C-reactive protein; **CRP*[c]**
DET1 partner of COP1 E3 ubiquitin ligase; **DET1**	Armadillo repeat--containing protein 2; **ARMC2**	Zinc finger protein 561; **ZNF561**
Protein polybromo-1; **PBRM1**	Caspase recruitment domain--containing protein 11; **CARD11**	Zinc Finger Protein 83; **ZNF83**
Defective in cullin neddylation 1 domain containing 2; **DCUN1D2**	Mini chromosome maintenance 8 homologous recombination repair factor; **MCM8**	Vacuolar protein sorting--associated protein 18 homolog; **VPS18**

Ethanolamine Kinase 2; **ETNK2**	Carbonyl reductase family member 4; **CBR4**	Nuclear Factor Of Activated T Cells 2 Interacting Protein; **NFATC2IP**
Protein lin-7 homolog C**;** **LIN7C**	Ligand-dependent nuclear receptor-interacting factor 1; **LRIF1**	Centrosomal protein of 41 kDa; **CEP41***
Leucine rich repeat containing 59; **LRRC59**	Microtubule associated serine/threonine kinase like; **MASTL**	Sp4 transcription factor; **SP4**
Interleukin 17 receptor B; **IL17RB**	Ubiquitin domain containing 2; **UBTD2**	Transforming growth factor beta receptor 2; **TGFBR2**

*genes related to the CNS diseases; ** genes with the role in neurodegenerative diseases; [a] genes with the documented role in AD; [b] AD - related genes involved in immune and inflammatory response; [c] common genes of miR - 146a and miR - 155

BIOGRAPHY

Isidora Rovčanin Dragović

She was born in 1987 in Mojkovac where she finished elementary school and then gymnasium in Podgorica. She graduated from the Medical Faculty in Novi Sad in 2012 with an average grade of 9.23 and defended her graduate–research paper with a grade of 10. She enrolled in Doctoral studies in 2013 at Medical Faculty in Podgorica and passed the exams with an average grade of 10.

She worked as a doctor at the Neurology Clinic of Clinical Center of Montenegro in 2015/16. She enrolled in Specialistic Studies of Neurology at the Medical Faculty in Belgrade in 2017. She was certified for neuropsychological assessment in 2019. She works as a teaching and research assistant at the Physiology department of the Medical Faculty in Podgorica.

For the Initial research, she studied the effect of magnesium on the peripheral nerve of an animal model. She participated in international projects, during which she was educated in the field of molecular-biological techniques and for work on cell culture, at research institutes in France and Italy. As a part of the national project of the Medical Faculty, she conducted a translational neuroscientific study for her Doctoral Thesis, through research into the roles of miRNA molecules in Alzheimer's disease. She independently conceived another research with a focus on Alzheimer's disease, a realization of which is ongoing.

She is the author and co-author of ten conference and four international journal publications, as well as an ad hoc reviewer in international journals. She is one of the editors of the leading international journal – „Journal of Alzheimer's Disease“

Awards: „Luča” awards for primary and secondary education; the best research presentation at the International Congress of Medical Students in Novi Sad, 2012; Award of the Ministry of Education of Montenegro for achieving an average of 10 in Doctoral studies; the best

presented work in the field of Alzheimer's disease and dementia at the World Neurology Conference on Controversies in Neurology, 2022.

She speaks English and German and uses Italian and Russian languages.

She is the mother of two children.

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BIOGRAFIJA

Isidora Rovčanin Dragović

Rođena je 1987. u Mojkovcu, gdje je završila osnovnu školu, a potom gimnaziju u Podgorici. Diplomirala je na Medicinskom fakultetu u Novom Sadu 2012. sa prosječnom ocjenom 9,23 i odbranila diplomski--istraživački rad sa ocjenom 10. Na Medicinskom fakultetu u Podgorici je 2013. upisala Doktorske studije i položila ispite sa prosječnom ocjenom 10.

Radila je kao ljekar Klinike za neurologiju Kliničkog centra Crne Gore, 2015/16. Specijalističke studije neurologije na Medicinskom fakultetu u Beogradu je upisala 2017. Sertifikovana je za neuropsihološku procjenu 2019. Zaposlena je na Katedri za fiziologiju Medicinskog fakulteta u Podgorici, gdje sprovodi praktičnu nastavu i naučno-istraživački rad.

U okviru Polaznog istraživanja je ispitivala uticaj magnezijuma na periferni nerv animalnog eksperimentalnog modela. Učestvovala je u međunarodnim projektima tokom kojih se edukovala iz oblasti biohemijskih i molekularno-bioloških tehnika, kao i za rad na ćelijskoj kulturi, u Institutu za istraživanje kancera i starenja u Nici i u Institutu za biomembrane i bioenergetiku u Bariju. U okviru nacionalnog projekta Medicinskog fakulteta, sprovela je translacionu neuronaučnu studiju za doktorsku disertaciju, kroz istraživanje uloga miRNK molekula u Alchajmerovoj bolesti. Samostalno je koncipirala još jedno istraživanje sa fokusom na Alchajmerovu bolest, čija je realizacija u toku.

Autor je i koautor 10 konferencijskih radova i 4 internacionalne žurnalske publikacije, kao i ad hoc recenzent u međunarodnim časopisima. Jedan je od urednika u vodećem međunarodnom časopisu – „Journal of Alzheimer's Disease".

Nagrade: diplome „Luča" za osnovno i srednje obrazovanje; najbolji istraživački rad na Internacionalnom kongresu studenata medicine u Novom Sadu, 2012; nagrada Ministarstva prosvjete Crne Gore za ostvareni prosjek 10 na Doktorskim studijama; najbolji prezentovan rad u oblasti Alchajmerove bolesti i demencija na Svjetskoj neurološkoj konferenciji o kontroverzama u neurologiji, 2022.

Govori engleski i njemački, služi se italijanskim i ruskim jezikom.

Majka je dvoje djece.

Bibliografija:

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Attachment 1.

Statement of authorship

Signed: Isidora Rovčanin Dragović, MD

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The title of the Doctoral Dissertation „A new method for stratification of the risk for Alzheimer's disease in patients in Montenegro"

Mentor: Nataša Popović, MD, PhD, Associate Professor at Faculty of Medicine, University of Montenegro

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Ime i prezime autora Isidora Rovčanin Dragović

Broj indeksa/upisa 1/13

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Naslov rada „Nova metoda za stratifikovanje rizika za obolijevanje od Alchajmerove bolesti kod pacijenata u Crnoj Gori"

Mentor: Prof. dr Nataša Popović,

Komentor: Prof. dr Milica Martinović

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UNIVERZITET CRNE GORE

MEDICINSKI FAKULTET

Isidora Rovčanin Dragović

NOVA METODA ZA STRATIFIKOVANJE RIZIKA ZA OBOLIJEVANJE OD ALCHAJMEROVE BOLESTI KOD PACIJENATA U CRNOJ GORI

DOKTORSKA DISERTACIJA – PROŠIRENI REZIME NA SLUŽBENOM JEZIKU

Podgorica, 2024.

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Datum odbrane:

17. 7. 2024.

Lektori

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Prof. Marina Šestović, profesorica crnogorskog jezika i književnosti

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Naslov doktorske disertacije: Nova metoda za stratifikovanje rizika za obolijevanje od Alchajmerove bolesti kod pacijenata u Crnoj Gori

Datum prijave doktorske teze: 23. 7. 2020. godine, br.1071.

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Uvod: Očekivani životni vijek se udvostručio od početka XX vijeka, što je dovelo do povećane incidence Alchajmerove bolesti (AB), kao bolesti starije životne dobi (1). Aktuelno, 50 miliona ljudi u svijetu je pogođeno ovom progresivnom i konačno fatalnom neurodegenerativnom bolešću. Uzimajući u obzir činjenicu da je starost vodeći faktor rizika za AB, očekivano je da će broj oboljelih dostići cifru od 139 miliona do 2050. godine (2). AB uzrokuje 60-70% slučajeva demencije, koje zauzimaju peto mjesto među uzrocima smrti u svijetu (12,13). Posljednji dotupni podaci ukazuju da je stopa mortaliteta od AB 2013. godine u Evropi, bila 45.2 na 100,000, odnosno, udvostručena u odnosu na 1994 godinu (14). Zvanični podaci za Sjedinjene američke države ukazuju da je AB bila šesti vodeći uzrok smrti 2019., odnosno sedmi u 2020. i 2021., zbog COVID-19 pandemije. Istraživanja ukazuju da ovaj globalni, rastući trend obolijevanja od AB, ne zavisi od socio-ekonomskog statusa, a ukupno finansijsko opterećenje zravstvenih sistema zbog ove bolesti, u rangu je sa onim za kardiovaskularne bolesti i karcinom (15). Takođe, troškovi za same oboljele od AB, smatraju se prevelikim (16). Zbog prirode ove bolesti, dodatno ih uvećavaju negativni ishodi koji se odnose na mentalno i fizičko zdravlje članova porodice (1,17,18), kao i broj sati posvećenih oboljelim od AB (1,15). Međutim, uprkos rastućem trendu morbiditeta, mortaliteta i opterećenja zdravstvenih sistema zbog AB, posljednje israživanje američke populacije ukazuje na nizak stepen svijesti o blagom kognitivnom poremećaju (BKP) (1), koji bi trebalo da upozori na potencijalni razvoj AB i da bude jasna indikacija za ekpertsku medicinsku konsultaciju.

Poznate činjenice o demografskom razvoju Crne Gore, upozoravaju da je globalni problem AB nesumnjivo izražen u našoj zemlji. Naime, trend demografskog starenja crnogorske populacije se bilježi već 50 godina, a procjenjuje se da će biti prisutan i do kraja prve polovine XXI vijeka. Pored toga, u Crnoj Gori zdravstvena statistika u oblasti AB još uvijek nije adekvatno implementirana. Monitoring i istraživanje statusa ovog dijela crnogorske populacije su primarno limitirani činjenicom da ne postoji zdravestveni registar za AB (22). Ipak, analizom podataka dostupnih u objavljenim izdanjima Statističkog godišnjaka Instituta za javno zdravlje Crne Gore, zaključuje se da je od 2016. do 2020. godine, oko 0,05% crnogorskog stanovništva godišnje, imalo dijagnozu AB (23), što je znatno manje u

poređenju sa drugim evropskim zemljama. Takođe, podaci o upotrebi ljekova u Crnoj Gori ne ukazuju na potrošnju onih namijenjenih za simptomatsko liječenje AB, a koji su aktuelno dostupni (25). Stoga, postoji ozbiljna zabrinutost da je za sada AB u Crnoj Gori nedovoljno dijagnostikovana i da bi broj oboljelih kojima je potrebna njega, mogao biti značajno veći.

Dešifrovanje složenog neurodegenerativnog procesa AB, kao i njegovog odnosa sa aspektima kliničkog sindroma, predstavlja vjerovatno najizazovniji aspekt istraživanja u oblasti AB. Do sada je nedvosmisleno dokazano da neuropatološki supstrat ove bolesti predstavljaju ekstracelularna akumulacija amiloid–β (Aβ) proteina i intracelularno taloženje tau preoteina (eng. aggregated tubulin associated unit – tau), kao i fosforilisanog tau proteina (p-tau) (29,31,32). Nakon niza genetskih i eksperimentalnih otkrića u familijarnim oblicima bolesti sa ranim početkom (eng. early onset Alzheimer's disease - EoAD), poremećaj metabolizma amiloida je ustanovljen kao početni fenomen u patofiziološkoj kaskadi AB (38,39). Tačnije, brojne mutacije u amiloid prekursor protein (APP), presenilin 1 (PSEN1) i presenilin 2 (PSEN2) genima detektovane sekvenciranjem (38,54,55), smatraju se dominantnim uzrokom deregulisane razgradnje APP i formiranja konačnog patološkog proizvoda - Aβ depozita (31,32,33,55). I kliničko-patološka i istraživanja aktuelnih biomarkera AB, ukazuju da akumulacija amiloida predstavlja veoma rani događaj, koji nastaje i decenijama prije kliničke manifestacije bolesti (44). Tau patologija je detektovana nešto kasnije, ali takođe deceniju ili više prije klinički evidentne AB (45). Prema amiloidnoj hipotezi o AB (AH), hiperfosforilacija tau proteina, oksidativni stres, neuroinflamacija, vaskularno oštećenje i konačno kognitivni pad za koje je dokazano da karakterišu AB, posljedice su patološke dinamike stvaranja i metabolisanja Aβ (33,34,51,52,63). Međutim, ova hipoteza ne nudi precizna objašnjenja o mehanizmima ili hronologiji događaja u patogenezi AB. Štaviše, nekim od prvih studija koje su ispitivale efikasnost redukcije amiloida u mozgu oboljelih od AB, nije demonstrirano poboljšanje kognitivnog statusa ispitanika (64). Međutim, uprkos evidentnim nejasnoćama AH, prisustvo amiloidnih depozita kao patomorfološkog supstrata AB nikada nije opovrgnuto, a prvi identifikovani biomarkeri ove bolesti se upravo zasnivaju na detekciji patoloških nakupina Aβ u mozgu (30). Ipak, nakon brojnih izazova u procesu istraživanja efikasnosti amiloid-redukujuće terapije (64),

klinička upotreba dva lijeka sa ovim mehanizmom djelovanja je konačno opravdana i odobrena u Americi, od strane Agencije za hranu i ljekove (eng. Food and Drug Administration – FDA) (68). Ovim činom, translaciona vrijednost AH jeste dokazana, međutim, mnoge studije nedvosmisleno ukazuju da ova teorija ne može samostalno objasniti složeni patofiziološki kontekst AB. U vezi sa tim, predloženo je nekoliko bioloških teorija AB, kao vodećih ili komplementarnih AH: transport i metabolizam lipida, intracelularni vezikularni transport, imuno-inflamatorni odgovor, angiogeneza, apoptoza, oksidativni stres i mitohondrijalna disfunkcija, homeostaza gvožđa i metabolizam kalcijuma (29,32,35,65,66). Već decenije istraživanja značaja ovih mehanizama, dokazuju dominaciju koncepta inflamacije, kao vodećeg objašnjenja AB (66). Njen značaj u neurodegenerativnom procesu AB zapažen je prije 30 godina, kada je objavljena studija o povezanosti hronične upotrebe antiinflamatorne terapije i smanjenja incidence i progresije AB (69). Pokazana je i pozitivna korelacije između nastanka demencije i podataka o prethodnim sistemskim infekcijama (70). Nakon toga, brojne eksperimentalne i kliničke studije potvrdile su ova početna zapažanja. Među važnijim dokazima su oni koji upućuju na povezanost gena koji nose povećan rizik od AB i statusa funkcija urođeng imuniteta (65,75), kao i korelaciju između incidence AB kasnog početka (eng. late onset AD – LoAD) i polimorfizama gena koji kodiraju ključne proteine urođenog imunološkog odgovora (76 ,77). Takođe, utvrđeno je značajno povećano prisustvo i aktivacija proinflamatornih citokina i hemokina u mozgu pacijenata sa AB (72,73), posebno u regijama nakupljenih Aβ peptida i neurofibrilarnih klubadi (83). Interesantni su rezultati istraživanja koji pokazuju da je aktivacija mikroglije, kao nosioca procesa neuroinflamacije (78,79), u inverznoj korelaciji sa zapreminom hipokampusa i metabolizmom glukoze kod pacijenata sa AB (86,87), a čak i sa njihovim kognitivnim performansama (88,89). Pored toga, nedavno sprovedene sudije ukazuju na to da bi neuroinflamacija mogla prethoditi ili čak generisati patološke amiloidne i tau kaskade (90,91,92), što je pozicionira ne samo u centar već i na početak patofiziološkog procesa AB. Dalje, pokazano je da neuroinflamatorni proces i specifični aktivirani citokini, utiču na produkciju i metabolisanje Aβ peptida, još uvijek nedovoljno jasno interferiraju sa procesima patološke tau akumulacije, a takođe određuju ranu progresiju AB (84,85,91,92,93). Konačno, smatra se da je uloga mikroglije veoma važna i u procesu disfunkcije i gubitka sinapsi,

karakterističnih za AB (94). Stoga se uloge imuniteta i neuroinflamacije u AB, mogu smatrati ključnim nosiocioma i povećanog rizika za ovu bolest, i čitavog patogenetskog procesa do degeneracije neurona. Iako još uvijek ne postoji jedinstveno objašnjenje patogeneze AB, teorija neuroinflamacije pruža prilično sveobuhvatno i fundamentalno objašnjenje patofiziološkog konteksta AB. Za sada se nedvosmisleno jedino može tvrditi da sinergističko djelovanje brojnih opisanih patofizioloških procesa ove bolesti, dovodi do distrofičnog, neurodegenerativnog procesa u neuronima, sve do njihovog potpunog gubitka (51,52). Uzrokujući atrofiju kao dominantnu strukturnu patološku promjenu mozga u AB, neurodegeneracija započinje u hipokampusu i progredira do parijetalnog režnja, što rezultira najprije oštećenjem epizodičnog, a potom i dugoročnog pamćenja, kao dominantnih kliničkih karakteristika AB (29-32).

AB predstavlja najčešći uzrok demencije među starijom populacijom. Iako se starost smatra glavnim faktorom rizika (1,2,3,29,30,95), AB se može javiti i ranije tokom života, tako da aktuelno postoje dvije glavne kategorije bolesti u zavisnosti od starosne dobi u kojoj počinje bolest: EoAD < 65 godina i LoAD > 65 godine (29,30,96). U oba oblika, specifične patološke karakteristike AB (AB-P) izazivaju čitav spektar kliničkih manifestacija (AB-C), koje su podmuklog početka i tipično podrazumijevaju progresivni gubitak pamćenja kao i kognitivnih funkcija koje uključuju jezik, vizuelno-prostorne i egzekutivne domene, do potpunog kognitivnog osiromašenje sa fatalnim ishodom (29,30,31,32,96). Međutim, pridruženi neuropsihijatrijski poremećaji kao i heterogena klinička prezentacija same AB su značajne i nerijetko dovode do dijagnostičkih dilema. Sa druge strane, često prisustvo višestrukih i različitih neuropatoloških promjena u ovoj bolesti, kao i preklapanje sa biološkim fenomenima normalnog starenja, doprinse i jednoj patofiziološkoj raznolikosti koja može uzrokovati nejasne kliničke prezentacije AB. Na kraju, poligenska priroda AB nosi svojevrsnu kompleksnost. Stoga, razumijevanje ove složene bolesti kao i njeno adekvatno dijagnostikovanje jeste izazov, koji je nametnuo potrebu i za evolucijom samog koncepta AB. Naime, nakon dugog perioda isključivo kliničkog pristupa, kada se dijagnoza AB razmatrala samo kod onih koji su ispoljili specifično kognitivno oštećenje (eng. cognitive impairment - CI) a njena potvrda bila moguća tek post mortem, biološki pristup omogućen

progresijom na polju biomarkera, doveo je do značajnog zaokreta u razumijevanju ove bolesti. Prije svega, sada je jasno da je AB dinamičan, kontinuiran i progresivan proces, od asimptomatske bolesti do demencije (30,105). Procjenjuje se da na početku kontinuuma AB, oko 30% osoba ima određeni nivo AB-P, ponekad čak i uznapredovale, ali bez očiglednih simptoma (106). Zapravo, neurodegeneracija nastaje postepeno, a kada se klinički manifestuje, bolest se prethodno razvijala najvjeroverovatnije nekoliko decenija (29,30,44,45). Jedno od objašnjenja ovakve prirode AB, moglo bi biti bazirano na bogatim dokazima o ulozi neuroinflmacije, a u svjestlu poznatog koncepta o razvoju niskog stepena inflamacije sa starenjem organizma, kao faktoru rizika za morbiditet i mortalitet (107). Naime, LoAD bi mogla biti shvaćena kao hronična bolest povazana sa starenjem, slično drugim poznatim bolestima koje se zasnivaju na hroničnom razvoju inflamacije, kao što su: kancer, dijabetes tip II i kardiovaskularne bolesti (107,108,109). To bi upravo moglo razjasniti postepenu akumulaciju neuropatoloških promjena u mozgu, kao i dinamičku prirodu AB, sa dugom pretkliničkom fazom. Objašnjenje kognitivne intaktnosti određenih individua uprkos razvoju AB-P, moglo bi se zasnivati na varijabilnosti bazičnih neurokognitivnih sposobnosti, odnosno, na konceptu rezerve (110,111). Fenomen moždane rezerve, podržava kogniciju u kvantitativnom smislu, odnosno, bazira se se na individualnom strukturnom kapacitetu i integritetu mozga, uključujući broj neurona i sinapsi. Sa druge strane, kognitivna rezerva (eng. Cognitive reserve - CR) se gradi tokom života i prema istraživanjima, njen kapacitet se povećava kroz: obrazovanje, kognitivno-stimulativne aktivnosti, složenost zanimanja koje se obavlja, poznavanje različitih jezika, a bazira se i na koeficijentu inteligencije i socio-ekonomskom statusu (110-113). Pokazano je da će se isti nivo AB-P kasnije manifestovati odnosno klinički dijagnostikovati kao AB, kod osoba sa većom moždanom i/ili CR, u poređenju sa osobama sa niskom rezervom (114,115).

Biološki markeri su omogućili da se prisustvo AB definiše u odsustvu kliničkih manifestacija (30) i stoga suštinski i značajno unaprijedili razumijevanje ove bolesti i otvorili nove istraživačke mogućnosti. Biološki koncept i definicija AB naglašavaju da klinička prezentacija – kognitivno oštećenje, predstavlja samo simptom ili znak bolesti, odnosno odraz jednog ili više neuropatoloških supstrata, čija se biološka priroda može spoznati samo

identifikovanjem prisustva biomarkera (30). Aktuelne biomarkere AB je najjednostavnije podijeliti prema metodološkom pristupu njihove detekcije: biohemijski, izolovani iz likvora i biomarkeri koji se detektuju neuroradiološkim metodama (118,119). Sa druge strane, uzimajući u obzir patološke procese koje identifikuju biomarkeri, klasifikuju se kao oni koji određuju akumulaciju Aβ, neurofibrilarnih tau depozita i stepen neurodegeneracije (120). Upravo određivanjem nivoa amiloida u cerebrospinalnoj tečnsti kao i njegovom detekcijom amiloid pozitron emisionom tomografijom (amiloid PET), dokazano je da se ova patologija u AB, javlja i 20 godina prije kliničke manifestacije bolesti (44,130,131). Prema aktuelnim kriterijumima, dovoljno je da se detektuje amiloidni AB neuropatološki supstrat, da bi se smatralo da su patološke promjene Alchajmerovog tipa (30). Međutim, pozitivan tau biomarker zajedno sa prisutnom akumulacijom amiloida, neophodan je da bi se potvrdila AB dijagnoza. Zapravo, u cilju adekvatnog definisanja stadijuma AB, kao i razumijevanja biološkog značaja prisustva svake od aktuelno poznatih patoloških promjena AB (odnosno njihove kombinacije), Nacionalni institut za starenje i Alchajmer asocijacija (eng. National Institute of Aging-Alzheimer's Association - NIA–AA) uspostavili su ATN sistem klasifikacije. Svako od tri navedena slova, indikuje specifičan patološki proces – amiloid („A“), tau patologija („T“) i neurodegeneracija („N“), koji može biti detektovan ili promjenama u CST ili neuroradiološki. To omogućava kreiranje specifičnog ATN profila za svakog pojedinca (30).

Iako individualni ATN profil biološki određuje AB kontinuum, on ne ukazuje nužno na ozbiljnost kliničkih manifestacija. Sa druge strane, za razliku od neuropatološkog supstrata otkrivenog biomarkerima, klinička prezentacija AB je nespecifična i stoga ne pruža mogućnost biološkg definisanja bolesti (5,6,132,134-137). Zapravo, pokazano je da isključivo klinički pristup AB ima skroman dijagnostički potencijal (143-154). Dokazano je da 25%-30% pacijenata pogrešno dijagnostifikovano kao AB demencija, nakon kliničke evaluacije u specijalizovanim klinikama za demenciju (144,145,146). Na nivou primarne zdravstvene zaštite, čak 50% - 70% simptomatskih slučajeva AB nije dobilo tačnu dijagnozu ili je ostalo neprepoznato (147,148,149,150). Među onima koji su bili podvrgnuti autopsiji, oko 10% - 30% slučajeva AB demencije, klinički dijagnostikovanih od strane eksperata, nije

imalo neuropatološke promene AB tipa (149,151). Konačno, rezultati istraživanja pokazuju da senzitivnost i specifičnost detekcije neuropatoloških AB promjena kliničkom evaluacijom iznose 81%, odnosno 73% (143,144,146). Međutim, uprkos jasnom, objektivnom ograničenju neurologa da se izjasni da je AB sindrom etiološki AB, bez biološke potvrde, aktuelni biomarkeri nijesu široko dostupni, za rutinsku kliničku primjenu. To je vjerovatno i glavni razlog fleksibilnosti zvaničnih dijagnostičkih kriterijuma, koji ne obavezuju kliničare da uključe informacije o aktuelnim biomarkerima u proces donošenja dijagnostičkih odluka u vezi sa AB (30). Dijagnostička procedura se neizbježno zasniva na kliničkoj evaluaciji i neuropsihološkoj procjeni kognitivnih performansi pacijenata sa AB (30). Prema zvaničnim dijagnostičkim i istraživačkim kriterijumima, **postoje tri glavne kategorije koje ukazuju na kognitivni status ili težinu kognitivnog oštećenja** i takođe predstavljaju svojevrsni kontinuum kliničke prezentacije AB: bez kognitivnog oštećenja, BKP i demencija (30,138-140,142). Očekuje se da će klinička procjena i neuropsihološko-kognitivna evaluacija za **osobe bez kognitivnog oštećenja** biti u standardizovanom opsegu normalnog. Međutim, ponekad se kognitivni deficit može identifikovati u odnosu na normu postavljenu za opštu populaciju, ali, uzimajući u obzir godine i obrazovanje, takav status za određenu osobu, može zapravo biti očekivan (138-140). Sa druge strane, imajući u vidu biološki kontinuum AB, slučajevi bez kognitivnog oštećenja, određeni kognitivnom evaluacijom, mogu zapravo pripadati pretkliničkom stadijumu AB, ukoliko bi se detektovalo prisustvo AB biomarkera (30). Suptilne kliničke manifestacije kognitivnog pada prije objektivnih dokaza – subjektivni kognitivni pad (eng. subjective cognitive decline - SCD) uzrokovan AB, predstavlja strukturisan (155) i široko prihvaćen koncept. Međutim, njegova rutinska primjena je ograničena, jer kriterijumi podrazumijecaju uključivanje i detaljnih anamnestičkih i heteroanamnestičkih podataka, kao i dokaze o prisustvu ApoE ε4 genotipa i AB biomarkera, kako bi se potvrdilo da je AB uzrok SCD (155). Ipak, isključivo klinička evaluacija subjektivnog kognitivnog statusa kroz pitanja otvorenog tipa, podržana je kao mogućnost zvaničnim SCD kriterijumima (155), i svakako je unaprijedila rutinsku kliničku praksu u smislu rane identifikacije mogućeg razvoja AB sindroma. Najraniji stadijum AB koji je dostupan objektivnoj procjeni aktuelno prihvaćenim dijagnostičkim metodama (30,139) je **BKP**, koncept koji je definisao Petersen sa saradnicima prije više od 30 godina (158). Prema

zvaničnim smjernicama, BKP je pretežno amnesičkog tipa ukoliko je AB vjerovatna biološka osnova tog sindroma (139). To znači da su najistaknutija klinička manifestacija kao i neuropsihološki nalaz, subjektivno i objektivno oštećenje epizodičnog pamćenja, odnosno sposobnosti učenja i zadržavanja novih informacija. Treba naglasiti da i drugi oblici BKP, koji podrazumijevaju kognitivno oštećenje u pojedinačnim domenima ili više njih, bez poremećaja memorije (159), takođe mogu biti uzrokovani AB. Kao rana faza AB kontinuuma dostupna standardizovanoj kliničkoj identifikaciji, BKP stadijum takođe predstavlja pogodnu i dragocjenu osnovu za istraživanje ranih patoloških procesa AB. Uspješna klinička **procjena progresije od BKP do sindroma demencije,** zasniva se na kontinuiranoj i ponovljenoj evaluaciji kognitivnog statusa i sposobnosti pacijenta u obavljanju aktivnosti svakodnevnog života (140). Iako su dijagnostičke smjernice utvrđene i jasne, za svakog pacijenta je neophodna procjena od strane kvalifikovanog kliničara, u kontekstu starosti, obrazovanja, opštih intelektualnih kapaciteta i komorbiditeta (30,140). Multidomenska amnestička demencija se smatra tipičnom kliničkom slikom AB, međutim, ovaj fenotip može biti uzrokovan i drugim bolestima. Sa druge strane, osnovni uzrok neamnestičkih prezentacija, egzekutivnih, jezičkih ili vizuelno-prostornih, takođe može biti posledica AB (30). **Sindrom AB demencije se dijeli na blagi, umereni i teški stadijum** (140). Veoma je važno imati na umu ovaj kognitivni kontinuum samog sindroma demencije, jer se otkrivanje njegove rane faze ipak može smatrati blagovremenim, u kontekstu aktuelnih terapijskih mogućnosti. U vezi sa tim, postoji koncept rane AB (eng. early AD - EAD), koji objedinjuje BKP i stadijum blage demencije (68,161,162,163,164,165). Naime, on je posljednje dvije godine dobio posebnu praktičnu vrijednost, kroz kriterijume za terapiju monoklonskim antitijelom – adukanumabom, prvom odobrenom amiloid-redukujućom terapijom, indikovanom u ranoj AB (68). Evolucija otkrića i saznanja o AB, dovodi nas do zaključka da se klinički i biološki aspekti bolesti ne mogu izjednačiti, stoga nas na izvjestan način i dalje ostavlja na nesigurnom kliničkom terenu. Drugim riječima, paradoks postojanja AB biomarkera koji jedino mogu donijeti biološku potvrdu ove bolesti, leži u činjenici da njihova upotreba nije dostupna u rutinskoj kliničkoj praksi.

Obećavajuća je činjenica da istraživanja i razvoj novih biomarkera značajno napreduju. Dominantan pravac predstavlja istraživanje biomarkera iz krvi, od kojih se očekuje da će imati kapacitet da prevaziđu glavne nedostatke aktuelnih AB biomarkera. Kako bi bili lako i široko dostupni, svakako bi otvorili mogućnosti skrininga i tako nas doveli do glavnog cilja, ranog otkrivanja AB. Osim toga, skupa i nedostupna (amiloid) PET metoda i invazivnost lumbalne punkcije, neophodnih za određivanje aktuelnih biomarkera, ograničavaju njihovu primjenu u opštoj populaciji. Međutim, uzorkovanje krvi i potom određivanje prisustva biomarkera, bili bi minimalno invazivni i pristupačni, što bi omogućilo i češća kontrolna mjerenja u cilju kliničkog, ali i praćenja u istraživanjima. Do sada je identifikovano nekoliko novih, potencijalnih biomarkera, koji bi reflektovali prisustvo amiloidne, tau patologije kao i neurodegenerativnog procesa mozga u AB, kroz detekciju njihovih nivoa u krvi: Aß, tau protein, svjetlo neurofilamenta (eng. neurofilament light - NFL) i glijalni fibrilarni kisjeli protein (eng. glial fibrilar acidic protein - GFAP) (166,167,168). Međutim, prema preporukama Alchajmerovog udruženja objavljenim 2022. godine, i pored nesumnjivih i brojnih prednosti svakog od pobrojanih, potencijalnih biomarkera, i dalje postoji potreba za tzv. head-to-head studijama koje bi testirale ove biomarkere u odnosu na već postojeće, longitudinalnim, kao i studijama u različitim populacijama, kako bi se potvrdila njihova efikasnost u različitim klinikim scenarijima. Takođe, neophodna su dodatna ispitivanja laboratorijskih testova koji bi bili potpuno adekvatni, uz uspostavljene univerzalne referentne vrijednostima, kako bi potencijalni biomarkeri ušli u širu upotrebu (174).

Dinamika istraživanja i napori usmjereni na terapijska rješenja za AB, u velikoj su suprotnosti sa aktuelnim mogućnostima za tretman ove, još uvijek neizlječive bolesti. Simptomatska terapija, koja je u upotrebi već 20-30 godina, i dalje predstavlja jedine dostupne tretmane u rutinskoj kliničkoj praksi. Prvi terapijski izbor, za koji je pokazano da poboljšava kognitivni status u ranim stadijumima demencije, predstavljaju agensi koji inhibiraju enzim acetilholinesterazu (29), dovodeći do povećane količine acetilholina (Ach) u sinapsama (29,193), čiji nedostatak dokazano kompromituje hipokampalnu i kortikalnu funkciju u AB (194). Pozitivni efekti antagonista N-metil-D-aspartata (NMDA), memantina,

zasnovani su na nalazu da toksičnost posredovana glutamatom i posljedično preplavljivanje kalcijumom, koji dovode do mitohondrijalne disfunkcije i visokog nivoa slobodnih radikala, uzrokuju degeneraciju odnosno gubitak neurona u AB (198). Nakon odobrenja memantina 2003. za umjerenu do tešku AB (29,193), punih 18 godina nije bilo novih ljekova za AB na tržištu. U vrijeme kada je prva imunoterapija odobrena 2021. godine (68), bilo je ukupno oko 130 agenasa za AB u fazama II ili III, sa zastupljenošću terapije koja bi modifikovala AB djelovanjem na amiloid ili tau patološki supstrat, u više od 70% kliničkih ispitivanja (67). Očekuje se da će adukanumab, kao amiloid-redukujući agens, pozitivno uticati na progresiju bolesti, a ne samo na ublažavanje simptoma (68). Treba napomenuti i da je istraživanje efekata ovog monoklonskog antitijela inicijalno bilo osporavano, jer nije dovelo do poboljšanja kognitivnih i funkcionalnih sposobnosti studijskih ispitanika (64). Međutim, upravo zahvaljujući tim izazovima, uvidjelo se da je ova vrsta terapije koja bi trebalo da modifikuje tok bolesti, trebalo da bude testirana ranije u patofiziološkom kontinuumu AB (199). Nakon primjene ovakve strategije, uspjeh je evidentan i ova terapija indikovana kod pacijenata sa ranom AB. Ipak, izbor oboljelih podobnih za ovu terapiju, kao i njena primjena, zahtijevaju ekspertski kadar, skupu i naprednu infrastrukturu, što nedvosmisleno ograničava njenu široku primjenu. Konačno, nakon pojave adukanumaba na američkom tržištu, FDA je odobrila upotrebu i drugog monoklonskog antitela – lekanemaba, početkom 2023. godine (202). U poređenju sa adukanumabom, ovaj lijek targetira složenije Aβ fragmente – protofibrile, za koje se smatra i da stimulišu povezivanje slobodnih monomera, što doprinosi širenju amiloidnih plakova (202,203). Stoga se pretpostavlja da će lekanemab imati višestruku i veću kliničku korist nego aducanumab. Iako je u oba slučaja riječ o tretmanu koji modifikuje bolest, ipak je namijenjen simptomatskim pacijentima, tako da još uvijek nije postignut cilj, da se, već pretklinički kompromitovana neurobiologija u ovoj bolesti, i tretira prije pojave prvih simptoma. **Sa tim ciljem, za sada se mnogo napora ulaže u istraživanje i razvoj preventivnih strategija.** Intervencije na polju životnog stila potrebne za sprječavanje razvoja AD ili odlaganje kognitivnog propadanja, proizlaze iz već bogatog znanja i dokaza o faktorima rizika za ovu bolest. Među njima, naglašava se značaj liječenja hipertenzije, lipidnog disbalansa i gojaznosti, kao i rješavanje problema gubitka sluha u srednjim godinama, kako bi se minimizirao rizik od razvoja AB (142,216-218,221,225).

Dokazano je da su podsticanje fizičke aktivnosti, socijalnih kontakata i prestanak konzumacije cigareta čak i posle 65. godine od posebnog značaja u ovom pogledu, zajedno sa liječenjem dijabetesa i depresije (209,214,221-223,226,227). Uvođenje mediteranskog tipa ishrane moglo bi da smanji rizik od konverzije amnestičkog tipa BKP u demenciju, prema nedavno sprovedenoj meta-analizi (221,227). Pored toga, za pojedinca koji je već biološki u AB kontinuumu, uticaj na brzinu razvoja bolesti do konačnog nastanka demencije, u velikoj meri zavisi od faktora rizika (228). Smatra se da oni koji nijesu nosioci APP/PSEN mutacija, imaju visok stepen kognitivne rezerve i niskorizični stil života, imaju kapacitet da čak kompenzuju biološke promjene izazvane AD-P i značajno odlože manifestaciju kognitivnog oštećenja (228). Značaj blagovremenog tretiranje onih faktora rizika na koje se može uticati oslikavaju i važni dokazi iz literature, koji ukazuju da takav pristup dovodi do prevencije vjerovatno jedne trećine slučajeva demencije (206,209). Stoga, savremeni čovjek mora imati razvijenu svijest o učestalosti i mogućnosti razvoja bolesti povezanih sa starenjem, uključujući i AB, odnosno, treba da vodi život u skladu sa idejom o njihovoj prevenciji već u četrdesetim godinama. Za sada je to i najbolje, potencijalno rješenje za AB.

Prethodno izloženo, nameće pitanja i potrebu za djelovanjem. Ukoliko biomarkeri nijesu dostupni za rutinsku primjenu u opštoj populaciji, postavlja se pitanje: kako možemo očekivati uspostavljanje rane dijagnoze AB? Uzimajući u obzir još i navedenu heterogenost i kompleksnost ove bolesti, kako možemo biti potpuno sigurni u ispravnost dijagnoze, čak i kod pacijenata sa kliničkim manifestacijama? Identifikovanje pacijenata koji se kvalifikuju za novu terpiju monoklonskim antitijelima indikovanu u ranoj AB, zahtijeva dostupnost sofisticiranog imidžinga, pa se nameće još jedno pitanje: kome su zapravo namijenjeni novi terapijski modaliteti? Aktuelno, namijenjeni su manjini pacijenata koji imaju priliku da budu evaluirani u velikim klinikama, specijalizovanim za ovu patologiju. Stoga, da li je uopšte realno očekivati široku primjenu ove terapije u budućnosti? Moglo bi se zaključiti da je identifikacija novih, široko dostupnih, jednostavnih i neinvazivnih biomarkera koji bi odražavali patofiziološki proces AB, urgentna. Oni bi omogućili skrining i rano otkrivanje AB, a takođe olakšali i popularizovali primjenu dostupne terapije koja modifikuje bolest. Konačno, oni bi mogli inspirisati istraživanje novih, uzročnih tretmana za AB.

Kakav je naučni kontekst ove studije, kao sredstva u potrazi za odgovorima? U mnoštvu istraživanja koja su preplavila oblast AB, sa glavnim zadatkom da identifikuju biomarker ranog stadijuma bolesti neinvazivnom, široko dostupnom metodom, postoji potreba za inovativnim pristupom istraživanju. Inovativnost i prednost ove studije zasnivaju se na njenom dizajnu. Naime, ponuđen je novi i širi istraživački kontekst za identifikaciju potencijalnog biomarkera AB. On je zasnovan na dokazima da dvije različite bolesti, AB i karcinom, imaju inverzne incidence i složene, većinom inverzne molekularne odnose. Male nekodirajuće RNK molekule - mikroRNK (miRNA), duboko su uključene u patogenezu obje bolesti i smatra se da imaju biološku kompleksnost kojom bi se mogla objasniti povezanost AB i kancera. **Ukratko, AB, kancer i miRNK su ujedinjeni kroz inovativni istraživački pristup, koji će potencijalno rezultirati otkrivanjem novog biomarkera AB.**

Dobro je poznato da su glavni patogeni mehanizmi AB i kancera suprotni po svojoj prirodi: AB karakteriše ćelijska smrt apoptozom, a kancer nekontrolisana ćelijska proliferacija (29,230). Stoga, mnogi patofiziološki mehanizmi, u vezi sa: fenomenom ćelijske smrti, supresijom rasta, signalnim putevima proliferacije, replikativnom besmrtnošću, reparacijom DNK, angiogenezom, kao i nekim genetskim faktorima, poput tumorskog proteina p53 (*TP53*), suprimirani su u jednoj, ali pojačano eksprimirani ili aktivni u drugoj bolesti (29,31,32,38,51,230,231,232,233). Iako je inflamacija široko prihvaćen, inicirajući i vodeći mehanizam obije bolesti (66,71,231,234,), njena uloga je kompleksna, stoga je na nivou signalnih puteva takođe pokazana inverzna deregulacija kod AB i kancera (235,236,237,238,239).

Poslednjih godina, zanimljivo, raste broj naučnih dokaza koji upućuju da su incidence AB i raka takođe u obrnutoj korelaciji (232,245-248). Naime, epidemiološke studije pokazuju da osobe sa istorijom raka imaju manji rizik za obolijevanje od AB i obrnuto. Nedavno sprovedena meta-analiza je potvrdila povezanost značajno smanjenog rizika od AB i istorije kancera, ne detektujući značajnu heterogenost analiziranih studija, što ide u prilog relevantnosti ovih dokaza (248). Interesantno je i istraživanje kojim je pokazano da je kod osoba koje su ranije imale dva karcinoma različitog porijekla, prosječna starost na početku AB veća, u poređenju sa pojedincima koji su ranije imali jedan karcinom ili nijesu bolovali

od te bolesti (249). Takođe, identifikovane su razlike među tipovima karcinoma, u kontekstu njegove inverzne incidence sa AB. Na primjer, velika Framingham studija je pokazala da je rizik od AB bio manji među preživjelima od karcinoma povezanih sa konzumacijom cigareta, u poređenju sa onima koji su preživjeli karcinome koji nijesu povezani sa pušenjem (245). Smanjena incidenca obolijevanja od kolorektalnog adenokarcinoma (CAC), koji je u fokusu ovog istraživanja, dokazana je kod pacijenata sa AB i obrnuto (235,246,252-254). Odluka da se da se u ovu studiju uključe baš oboljeli od CAC - među svim kacinomima za koje je pokazana inverzna korelacija sa AB, nije bila vođena samo činjenicom da je CAC treći najčešće dijagnostikovan karcinom (255), već i kriterijumima za uključivanje u studiju, njenim dizajnom i izvodljivošću. Na primjer, za razliku od karcinoma pluća, koga najčešće karakteriše brža progresija i lošija prognoza, kod oboljelih od CAC bolest progredira obično sporo, prognoza je bolja, a ukoliko se dijagnoza postavi rano, nekada nijesu potrebne radio- i hemioterapija, koje bi mogle uticai na gensku ekspresiju. Precizni mehanizmi koji se nalaze u osnovi ove negativne epidemiološke povezanosti između AB i CAC su istraživani (258), ali još nijesu dovoljno rasvijetljeni. Transkriptomske meta analize, na primjer, otkrile su suprotnu regulaciju brojnih gena zajedničkih za AD i rak (259). Takođe, mnogi signalni putevi odgovorni za patogenezu obije bolesti, identifikovani su kao inverzno disregulisani kod AB i kod različitih tipova raka (232,233,235,237,250,252,260-265). Ipak, može se zključiti da su geni i putevi povezani sa inflamacijom, vjerovatno dominantni, među onima koji su zajednički za ove dvije bolesti (234-236,238,239,266).

Rezimirano, novija saznanja o inverznoj incidenci obolijevanja od AB i CAC, donijela su drugačiji i izazovan kontekst prethodnom znanju o zajedničkim biološkim mehanizmima ovih bolesti. Budući da su uglavnom suprotni po svojoj prirodi, pretpostavljeno je da ovi mehanizmi zapravo mogu predstavljati suprotne strane iste patofiziološke kaskade, objašnjavajući tako i suprotne incidence AB i CAC. Drugim riječima, ukoliko disregulacija nekog od zajedničkih AD – CAC signalnih puteva dovede do većeg rizika od kancera, pogodovaće smanjenom riziku za obolijevanje od AB kod iste osobe, ili obrnuto.

U potrazi za molekularnim objašnjenjem odnosa ove dvije bolesti, kao i za neinvazivnim i kliniki široko primjenljivim biomarkerom AB, **identifikovan je značajan broj**

molekularnih markera u serumu ili plazmi, uključujući miRNK (166-191,267-269). MiRNK su male, endogene, visoko konzervirane, nekodirajuće RNK molekule (270,271). Njihova važna uloga se sastoji u regulaciji ekspresije i translacije onih gena koji kodiraju proteine (270,271). Procijenjeno je da oko 1-4% ljudskog genoma kodira ove molekule, duge 19-25 nukleotida (272). MiRNK ostvaruju negativanu regulaciju ekspresije gena, kroz inhibiciju translacije ili potpunu degradaciju mRNK, što se smatra kanonskim, glavnim načinom delovanja ovih molekula (270-276). Podaci iz literature govore da svaka miRNK reguliše ekspresiju više od sto transkripata (272), čime utiče na do 30% gena koji kodiraju humane proteine (272,273). Jednostavna detekcija u tkivima i tjelesnim tečnostima (281) predstavlja dodatnu vrijednost ovih molekula i doprinosi njihovom translacionom potencijalu. Štaviše, miRNK u plazmi su stabilne molekule, koje se mogu identifikovati i kvantifikovati korišćenjem široko dostupnog metoda lančane rekacije polimeraze (PCR) (281).

Uticaj miRNK na glavne aspekte patogeneze i AB i kacera je nedvosmisleno pokazan (295). Glavne patološke karakteristike ovih bolesti su ćeljska smrt apoptozom, odnosno nekontrolisana ćelijska proliferacija (258). Kao dobro dokumentovani, vodeći regulatori ćelijske proliferacije i diferencijacije, migracije i apoptoze, miRNK bi mogle predstavljati ključne tačke patofiziološkog procesa obije bolesti (271-273,285,286,288,289). Stoga su upravo uloge ovih molekula značajno istraživane, kako bi se razumjelo da li je inverzna incidenca AB i CAC odraz disregulacije njihovih zajedničkih signalnih puteva (295,296). Literatura je bogata dokazima o posredovanju različitih miRNK u inflamaciji, oksidativnom stresu, mitohondrijalnoj disfunkciji, angiogenezi, vaskularnoj i endotelnoj disfunkciji, i kod AB i kod karcinoma (267-269,290,291,295-298). Osim toga, upravo navedeni procesi predstavljaju okosnice različitih teorija AB (29,32-34,51,63,66,73,77,82,90), tako da je proučavanje uloga miRNK u njenom nastanku i progresiji, prepoznato kao obećavajući istraživački pravac. Štaviše, dokazi koji upućuju da neke od miRNK posreduju u više različitih patofizioloških mehanizama AB ne iznenađuju, budući da svaka miRNK istovremeno djeluje na mnogo različitih targeta, često uključenih u različite biološke puteve (292,293). U tome se i ogleda njihov poseban kapacitet, kao potencijalno ključnih regulatora

patogeneze AB, a jedan broj miRNK molekula su već i prepoznate kao potencijalni biomarkeri ove bolesti (267-269,290,291,294). Sa druge strane, u kontekstu molekularnog odnosa AB i CAC, značaj miRNK je uglavnom demonstriran nezavisno u AB ili u CAC. U vrijeme kada je ovo istraživanje započeto, bilo je veoma malo objavljenih istraživanja sa fokusom na uloge miRNK u kontekstu molekularne, inverzne povezanosti ove dvije bolesti.

Uzimajući u obzir raznovrsnost i mnogostrukost uloga miRNK molekula, kao i širinu konteksta ovog istraživanja, bio je veoma izazovan zadatak odabrati miRNK koje će biti analizirane. Sve navedeno, naime, nametnulo je potrebu da se izbor miRNK, suzi na one koje su uključene u manji broj patoloških aspekata zajedničkih za AB i CAC. Smatralo se da će takav pristup doprinijeti temeljnijoj analizi, povećati mogućnost otkrivanja važnih molekularnih interakcija između AB i CAC i potencijalno identifikovati molekularni potpis ovog odnosa kroz specifičan obrazac ekspresije odabranih miRNK. Dominantne teorije obije bolesti, zasnivaju se na dva usko povezana procesa – urođenom imunitetu i inflamaciji, te su stoga i izabrani kao patofiziološki fokus ove studije. Uzimajući u obzir da su patogeneza i progresija AB posebno određene neuroinflamacijom i faktorima imunološke odbrane (66,71-75,78-89,92-94,108), pretpostavljeno je da će ovakav prstup takođe pomoći da se detektuju one molekule koje imaju ključni značaj u AB i potencijal da postanu biomarkeri. Stoga, svaka od odabranih miRNK za prezentovano istraživanje, ne samo da ima dokumentovanu ulogu u AB i CAC, već ima najmanje jedan target uključen u imunološke i/ili inflamatorne puteve: miR-29a, miR-101, miR -125b, miR-146a i miR-155 (299-333). Pored toga, mogućnost korišćenja ovih molekula kao neinvazivnih biomarkera, doprinosi posebnoj translacionoj vrijednosti cijelog istraživačkog konteksta predstavljenog ovdje.

Cilj: Ekspresija cirkulišućih miRNK, kompleksno uključenih u patogenezu AB i kolorektalnog carcinoma (CAC): miR-29a/b, miR-101, miR-125, miR-146a and miR-155, ispitivana je kod zdravih pojedinaca, onih sa blagim kognitivnim deficitom - verifikovanim skrining neuropsihološkim testovima, kao i kod pacijenata sa dijagnostikovanim AB i CAC. Postavljena je hipoteza da će ekspresija navedenih miRNK biti u korelaciji sa stepenom kognitivnog deficita ispitanika, što bi moglo biti od značaja u identifikovanju molekularnog potpisa rane AB. Takođe, očekuje se da bi obrazac ekspresije selektovanih miRNK kod

pacijenata sa AB i CAC, mogao objasniti molekularnu osnovu inverzne incidence ove dvije bolesti.

Metodologija: U ovu studiju su uključena 54 ispitanika, od ukupno 75 regrutovanih pojedinaca. Od 34 volontera koji su se osjećali fizički i mentalno zdravim, njih 18 je bilo uključeno u kontrolnu grupu (CTRL-18). Kod 9 učesnika koji subjektivno nijesu imali kognitivni deficit, kognitivne performanse na MoCA testu su bile u rasponu za MCI (eng. low-performance MoCA (LP-MoCA)-9). Pacijenti kod kojih je dijagnostikovana AB regrutovani su retrospektivno, u Klinici za neurologiju Kliničkog centra Crne Gore (KCCG) (AD-12). Ovim ispitanicima je dijagnoza prethodno postavljena u skladu sa kriterijumima Nacionalnog instituta starenja i Alchajmerove asocijacije. AD grupa je uključivala pacijente u ranom simptomatskom stadijumu bolesti (early Alzheimer's disease – EAD) – MCI uzrokovan AB i stadijum blage demencije (MoCA skor $\geq$ 17) i uznapredovaloj AB (advanced Alzheimer's disease – AAD) – umjerena i teška demencija (MoCA score < 17). Pacijenti sa CAC i bez kognitivnog oštećenja sa patohistološki potvrđenom dijagnozom, bili su regrutovani u Centru za digestivnu hirurgiju KCCG (CAC-15). Standardizovani upitnik, fizikalni i neurološki pregledi, neuropsihološki skrining testovi, gerijatrijska skala depresije, kao i biohemijska laboratorijska procjena, sprovedeni su sa ciljem identifikovanja isključujućih kriterijuma. Odabrane miRNK su bile izolovane iz plazme, kvantifikovane metodom lančane rekacije polimeraze u relnom vremenu i nivoi ekspresije normalizovani upotrebom miR-361-5p gena.

Rezultati: Ovom studijom su u kontrolnu grupu uključeni volonteri bez subjektivnog kognitivnog deficita, ali interesantno, na osnovu skora na MoCA testu, 33,3% njih, naizgled zdravih ispitanika, pripadalo je kategoriji MCI. Neurološki pregled i laboratorijske pretrage nijesu mogli objasniti učinak ovih pojedinaca na neuropsihološkim testovima. Međutim, u LP-MoCA grupi, nivo ekspresije dviju cirkulišućih miRNK, miR-146a i miR-155, bio je značajno viši u odnosu na kontrolnu ($p < 0.05$). Ekspresija miR-146a je bila u značajnom porastu u LP-MoCA i u odnosu na AD grupu ($p < 0.05$). U odnosu na EAD grupu, rezultati su pokazali isti obrazac ekspresije za obje miRNK - miR-146a i miR-155, kao i u odnosu na čitavu, heterogenu AD gupu. Razlika u nivou ekspresije ovih miRNK između EAD i AAD

grupa nije bila značajna. Konačno, analiza krivulje operativnih karakteristika (eng. Receiver operative characteristic curve – ROC curve), upućuje da bi miR-146a i -155 mogle služiti kao neinvazivni biomarkeri ranog kognitivnog oštećenja.

Među pet ispitivanih miRNK, samo je nivo ekspresije miR-101 u studijskim grupama bio u saglasnosti sa postulatom o inverznom odnosu između AB i CAC. Srednje vrijednosti ekspresije miR-101 za CAC i AD grupe bile su na suprotnim stranama opsega, dok je nivo ekspresije CTRL grupe bio u sredini (AD – 1.569, CTRL – 1.171 and CAC – 0.8340). Štaviše, ekspresija miR-101 bila je značajno veća u AD, u poređenju sa CAC grupom ($p < 0.05$). Nivo ekspresije ove miRNK u CTRL grupi se nije razlikovao u odnosu na AD i CAC grupe. Međutim, rezultati analize ROC krive upućuju na to da će u prosjeku CAC pacijent imati nižu vrijednost ekspresije miR-101 nego 64% zdravih pojedinaca, a AD pacijent bi imao povišenu regulaciju miR-101, u poređenju sa 63% opšte populacije. Ovom analizom je obezbijeđen kvalitativni skor, kojim je performansa ovog AD-CAC modela baziranog na ekspresiji miR-101, procijenjena kao dobra. Model je takođe testiran i u odnosu na stadijum bolesti AD grupe. Interesantno, ekspresija miR-101 bila je značajno veća u EAD u poređenju sa CAC ($p < 0.05$). Vrijednosti su se smanjivale sa progresijom AB, približavajući se nivou ekspresije CAC grupe, stoga nije opservirana značajna razlika između AAD i CAC grupa.

Diskusija: Rezultati prikazanog istraživanja su imali dva značajna aspekta. Prvim dijelom rezultata predstavljeno je ispitivanje prve dvije hipoteze - u vezi sa značajem proučavanih miRNK u AB. Najznačajniji rezultat predstavlja detekcija značajno većeg nivoa ekspresije miR-146a i miR-155 kod ispitanika čiji učinak na MoCA testu implicira da imaju BKP, u poređenju sa zdravim osobama i pacijentima sa AB. U drugom dijelu istraživanja, kojim je testirana treća hipoteza o inverznoj povezanosti AB i CAC, detektovan je inverzan nivo ekspresije miR-101 između AD i CAC studijskih grupa.

Prema našim saznanjima, BKP otkriven neuropsihološkim skriningom kod zdravih ispitanika koji nijesu imali SCD, do sada nije identifikovan. SCD je poznat kao fenomen subjektivne percepcije kognitivnog pada a kada objektivno oštećenje kognicije još uvijek nije prisutno, i može predstavljati prvu manifestaciju AB (155,156). Mnoge studije su pokazale značaj SCD za rano predviđanje razvoja klinički manifestne AB (30,155,156,346,347,348).

Rezultat ove studije pokazuje da je MoCA skor ovih volontera, koji je bio u BKP opsegu, u pozitivnoj korelaciji sa godinama njihovog školovanja ($r = 0,491$; $p < 0,05$). To bi moglo objasniti neadekvatnu subjektivnu percepciju kognitivnog deficita kod ovih ispitanika, sugerišući da bi niže obrazovanje moglo biti razlog nerazumijevanja da promjene kognitivnog statusa mogu biti znak narušenog zdravlja.

Zanimljivo je da je LP-MoCA grupa imala značajno više nivoe ekspresije miR-146a i miR-155, u poređenju sa zdravim ispitanicima kontrolne grupe. Različite studije sprovedene na ljudskim ili životinjskim modelima i ćelijskim kulturama tokom posljednjih deset godina, nedvosmisleno su dokazale značaj miR-146a i miR-155 u patogenezi AB (300-302,308-310,351). Povećan nivo ekspresije cirkulatorne miR-146a detektovan je kod pacijenata sa BKP koji su progredirali do AB, u poređenju sa onima koji su imali stabilan BKP (351). Takođe, viši nivo ekspresije miR-146 je pronađen kod nosilaca ApoE E4 genotipa, a ovaj nalaz je korelirao i sa neuroradiološkim markerima AB, kao i povećanom koncentracijom Aβ u likvoru (351). Interakcija između miR-155 i gena povezanih sa BKP, prikazana je u studiji Strafella i saradnika, koja je uz to otkrila i značajnu interakciju signalnih puteva miR-146 i miR-155 u patofiziološkoj kaskadi AB (354). Ukupno, ovi nalazi sugerišu da bi AB mogla biti u osnovi slučajno otkrivenog BKP kod dobrovoljaca u našoj studiji, koji su u odnosu na kontrolnu grupu, imali povećanu ekspresiju miR-146a i miR-155.

Konkretnije razumijevanje uloga miR-146a i miR-155 u određenim patofiziološkim putevima AB, dalje objašnjava potencijalni značaj njihove povećane ekspresije u LP-MoCA grupi. MiR-146a je poznata po svojoj ulozi u modulaciji urođenog imunološkog odgovora i inflamatornih događaja u moždanim ćelijama (300-302). U novijim studijama se navodi da je ova miRNK od izuzetnog značaja u neuroinflamatornim kaskadama AB (351,354-356). Značaj miR-155 u inflamatornim putevima AB, takođe je potvrđen istraživanjima (308-310,354,355). Sa druge strane, dokazi jasno upućuju da je neuroinflamacija najvjerovatnije inicijalna i ključna komponenta u patofiziološkoj kaskadi AB (66,92,360,361). Aktivacija mikroglije, koja je ključni medijator neuroinflamacije među ćelijama urođenog imuniteta, registrovana je prije formiranja patoloških plakova na animalnom modelu AB (360), ali i neuroradiološki, kod pojedinaca sa BKP (92,361). Svi ovi podaci idu u prilog hipotezi da se

povećanje nivoa ekspresije, sa inflamacijom pvezanih – miR-146a i -miR-155 kod ispitanika LP-MoCA grupe, može objasniti njihovom ulogom u ranim inflamatornim procesima AB.

Rezultati ovog istraživanja takođe pokazuju da nije bilo statistički značajne razlike u nivoima ekspresije miR-146a i miR-155 između kontrolnih i AD ispitanika, ali zanimljivo, miR-146a, je bila u povećanoj regulaciji u LP-MoCA, i u poređenju sa AD grupom. Još detaljniji uvid u neuroinflamatorne događaje AB i angažovanje miR-146a i miR-155 u tim mehanizmima, mogao bi ponuditi objašnjenje ovog rezultata. Naime, iako je u suštini odbrambeni, imuni odgovor može izazvati štetne posljedice, ukoliko je previše snažno indukovan ili predugo traje (362,363). Poznato je da upravo takav imuni odgovor karakteriše AB i dovodi do aktivacije homeostatskih mehanizama, u cilju ograničavanja destruktivnih inflamatornih događaja (364,365). Pokazano je da je jedna od uloga miR-146a upravo u supresiji patološkog neuroinflamatornog odgovora u AB. Soga bi se moglo zaključiti da je porast miR-146a ekspresije indukovan proinflamatornim citokinima (357,366,367), sa ciljem homeostatske, negativne regulacije gena - odnosno proteina u pretjerano aktivnim neuroinflamatornim signalnim putevima, kako bi se ograničila inflamacija (355). Shodno tome, moguće je da kroz mehanizam negativne povratne sprege, smanjen intenzitet inflamacije konačno dovodi i do smanjenja ekspresije miR-146a. Ovakvo razmatranje je u skladu sa rezultatima istraživanja na AB ćelijskoj kulturi, koje je pokazalo da su miR-155 i miR-146a bili visoko eksprimirani u mikroglija ćelijama tokom odgovora na prisustvo Aβ kao stresogenog faktora. Štaviše, nakon porasta nivoa inflamatornih citokina u tom odgovoru, uslijedilo je smanjenje ekspresije miR-146a, dok je registrovana perzistentna povećana regulacija miR-155 (368). Slična vremenska diskrepanca u promjeni ekspresije miR-146 i miR-155 tokom inflamatornog odgovora, potvrđena je i na životinjskim modelima (370). Konačno, ovi rezultati su u skladu sa nalazima nivoa ekspresije miR-146a i miR-155 u ovoj studiji, koja ima klinički kontekst. Dakle, značajan porast ekspresije miR-146a u LP-MoCA u poređenju sa kontrolnom grupom, korespondira sa njegovom dominantnom ulogom u ranoj inflamatornoj fazi AB, kroz interakciju sa medijatorima upale. Ponovna normalizacija miR-146a ekspresije do kontrolnih nivoa, vjerovatno odražava njenu supresiju homeostatskim, antiinflamatornim mehanizmima, karakterističnim za hroničnu fazu bolesti.

Sa druge strane, detektovani nivoi ekspresije miR-155 u ovoj studiji, impliciraju njegovu perzistentnu aktivnost, kao odraz kontinuiranog angažovanja u hroničnom inflamatornom procesu AB. Takođe, analiza ROC krivulje jasno sugeriše potencijalni dijagnostički značaj ovih miRNK, za skrining BKP. Osim potencijala ovih miRNK da diferenciraju pojedince koji su u riziku od razvoja manifestacija AB od objektivno zdrave populacije, poređenje CTRL, LP-MoCA i EAD grupa, ukazuje da se na osnovu ekspresije miR-146a takvi pojedinci mogu izdvojiti od populacije sa već postavljenom dijagnozom AB, uključujući i njenu ranu fazu. Navedeni rezultati naglašavaju potencijal za upotrebu ove miRNK kao skrining testa, odnosno njen potencijal za detekciju bolesti prije nego što su ispunjeni klinički kriterijumi.

Prethodno diskutovani, prvi dio rezultata, ne podržava prvu hipotezu. Nivoi ekspresije ispitivanih miRNK nijesu bili u korelaciji sa nivoom kognitivnog deficita kod pacijenata sa AB. Štaviše, isto se može zaključiti i kada se uzme u obzir LP-MoCA grupa, koja je neočekivano nastala tokom procesa istraživanja. Međutim, upravo specifičan obrazac ekspresije istraživanih miRNK koji je otkriven među ispitivanim grupama i nije korelirao sa nivoom kognitivnog oštećenja, potvrđuje drugu hipotezu. Kao što je navedeno, miR-146a i miR-155 izolovani iz plazme, mogli bi da identifikuju ljude sa kognitivnim oštećenjem koji su u riziku od AB, čime bi mogli biti od izuzetnog značaja u skriningu opšte populacije.

Diskusija drugog dijela rezultata, bazira se na razmatranju uloga miR-101 u kontekstu AB i AB – CAC odnosu. Najvažnija fiziološka uloga **miR-101,** koja je najviše proučavana u kontekstu AB je regulacija ekspresije *APP* gena (315,433). Smatra se, da se na toj ulozi i zasniva posredovanje miR-101 u patogenetskom procesu AB (315,316,433). *APP* je jedan od rijetkih dominantnih autozomnih gena, čije su različite alteracije nedvosmisleno povezane sa AB (29,38,39,52,55,59,102), dovodeći posljedično do povećanja ekspresije *APP* u ovoj bolesti (66,439,440,441,442). Osim toga, čak i kada AB ne karakterišu nužno mutacije *APP* gena, kao u LoAD, otkriveno je da je prekomjerna *APP* ekspresija izazvana drugim faktorima, kao što su neuroinflamacija (66,91,93,300,439,440) i mitohondrijalna disfunkcija (300,441,442). Mehanizmi povećane regulacije *APP* u AB su različiti, ali sa istom posljedicom - povećanom produkcijom APP zajedno sa patološkom dinamikom i

modifikacijama njegovog metabolizma, koji rezultiraju povećanjem produkcije Aβ, do toksičnih koncentracija (29-34,52,55,443).

MiR-101 negativno reguliše ekspresiju *APP* gena (315,433,445-447). Po principu komplementarnosti, ona stupa u interakciju sa 3′ UTR ciljnog transkripta mRNK, dovodeći do njene destabilizacije i/ili inhibicije translacije (271,272,285-287,313). U skladu sa ovim fiziološkim djelovanjem miR-101, istraživanja su pokazala da blokiranje njene interakcije sa *APP* genom, dovodi do povećanja njegove ekspresije (445). Takođe, pokazano je da inhibicija djelovanja miR-101 u kulturi hipokampalnih neurona dovodi do intenzivnijeg, patološkog metabolizma APP (433,446,447). Mnogo studija sa različitim metodološkim pristupom je sprovedeno sa ciljem istraživanja uloga miR-101 u AB (316,319,448,449) i rezultati su uglavnom u saglasnosti, ukazujući da je nivo ekspresije ove miRNK smanjen u AB (316,448,449). Pošto ovu bolest karakteriše prekomjerna ekspresija *APP* gena, smanjenje miR-101 povezano sa AB, dominantno se interpretira kao doprinoseći faktor. Naime, pretpostavlja se da nedostatak njenog prirodnog, inhibitornog uticaja na *APP* gen, dovodi do prekomjerne *APP* ekspresije u AB, sa posljedičnom patološkom modifikacijom APP metabolizma APP, do toksične akumulacije Aβ.

Za razliku od dokaza ponuđenih u literaturi, u prezentovanoj studiji postoji trend porasta nivoa ekspresije miR-101 kod pacijenata sa AB u odnosu na opštu populaciju, kao i značajno povećana ekspresija miR-101, u poređenju sa CAC populacijom pacijenata, za koju je poznato da ima smanjenu vjerovatnoću razvoja AB. Nalazi ovog istraživanja mogli bi se objasniti drugačije u odnosu na aktuelno prihvaćeno, vodeće stanovište. Kako je jedno od glavnih obilježja AB akumulacija Aβ, kao rezultat patološkog APP metabolizma (29-34,52,55-59,443), pretpostavljeno je da bi negativna regulacija ekspresije *APP* gena koja bi se postigla upravo povećanjem miR-101 ekspresije, mogla predstavljati odbrambeni mehanizam. Prema našim saznanjima, drugi istraživači nijesu pokazali porast ekspresije miR-101 kod pacijenata sa AB. Dalje, rezultati poređenja nivoa ekspresije miR-101 između EAD ispitanika - u ranoj AB i CAC pacijenata, ukazuju na značajno povećanje njene regulacije u ovoj AB podgrupi, u odnosu na CAC populaciju. Međutim, zanimljivo je da je trend ekspresije miR-101 sa progresijom bolesti bio opadajući,

približavajući se nivou ekspresije u CAC grupi. Stoga nije detektovana značajna razlika između AAD i CAC grupa, što implicira da pacijenti u uznapredovalom stadijumu AB ne doprinose značajnoj razlici opserviranoj između AD i CAC grupa. Zaključuje se, da nakon pokušaja organizma da se suprotstavi intenzivnom i toksičnom metabolizmu APP, kroz povećanje ekspresije miR-101, slijedeće smanjenje njene regulacije, može biti logična posljedica progresije bolesti. Naime, kako se proizvodnja Aβ povećava, uvodeći neurone u degenerativni proces, može se pretpostaviti da se inicijalno intenzivan metabolizam APP koji je potenicjalno indukovao miR-101 up-regulaciju, sada smanjuje, uzrokujući smanjenje miR-101 ekspresije. Dakle, moguće je da ukupno djelovanje ove miRNK treba shvatiti kao dio mehanizma negativne povratne sprege. U prilog takvom gledištu, utvrđeno je da je APP metabolizam povećan posebno u ranoj AB (458). Osim toga, status sinaptičke transmisije, koja je značajno kompromitovana neurodegenerativnim procesom u AB (29,31,32,51,460), može uticati na metabolizam APP (459). Stoga se kao logično nameće razmatranje, da progresija AB, akumulacija Aβ i posljedično oštećenje sinaptičke funkcije, mogu biti razlog demonstriranih promena u dinamici APP metabolizma, između ranih i kasnih stadijumima bolesti (458). Dakle, inicijalno povećana regulacija miR-101, kao regulatora *APP* ekspresije, može biti uzrokovana intenzivnim metabolizmom APP, ali sa njegovim smanjenjem u kasnijim fazama, i ekspresija miR-101 se smanjuje kroz mehanizam negativne povratne sprege. Zanimljiva studija koja je istraživala ulogu miR-384 u AB, koja je, kao i miR-101 negativan regulator *APP* gena, dokazala je upravo interakciju amiloida i miR-384 kroz mehanizam negativne povratne sprege (461). Kada su u pitanju klinički dokazi koji bi mogli biti relevantni u kontekstu predloženog mehanističkog koncepta regulacije miR-101 u AB, pronađena je negativna korelacija između gustine amiloidnog plaka kod pacijenata sa AB i nivoa ekspresije miR-101 u likvoru i serumu (462). Zapažanje da smanjenje ekspresije miR-101 u likvoru pacijenata sa AB korelira sa povećanjem gustine plaka, shvaćeno je kao dokaz doprinoseće uloge miR-101 bolesti. Međutim, pogled iz drugog ugla i mogućnost da se miR-101 ekspresija postepeno smanjuje i kao posljedica smanjenog APP metabolizma i akumulacije Aβ, vrijedna je ozbiljnog razmatranja. Štaviše, taj kontekst uzročne uloge miR-101, zajedno sa činjenicom da se Aβ akumulira tokom dugotrajne pretkliničke faze AB, prije implicira da bi ova miRNK već trebalo da bude smanjena u trenutku dijagnoze bolesti. Stoga

izgleda da se ova dinamička komponenta negativne korelacije između gustine plaka i ekspresije miR-101, zapravo bolje uklapa u hipotezu negativne povratne sprege. Konačno, djelovanje miR-101 u okviru mehanizma negativne povratne sprege, već je opisano u studiji Jing-a i saradnika, u kontekstu patogeneze CAC (463).

Predstavljeni rezultat ekspresije miR-101, implicira i potencijalnu kliničku primjenu. S obzirom na to da bi fluktuacija njene ekspresije mogla odražavati dinamiku Aβ akumulacije, miR-101 bi mogla biti ne samo indikator progresije bolesti, već i služiti za praćenje efekata nedavno odobrene amiloid-redukujuće terapije, indikovane u ranoj AB (202,465). Potvrda prisustva amiloidne patologije PET amiloid neuroimidžingom, neophodan je kriterijum za selekciju pacijenata za ovaj tretman (68). Rezultat ove studije ukazuje na to, da bi se normalan ili povećan nivo miR-101 ekspresije vjerovatno detektovao u ranom stadijumu akumulacije amiloida, a trend njenog smanjenja u uznapredovaloj bolesti, može odražavati progresiju amiloidne patologije. Stoga bi se minimalno-invazivnom detekcijom miR-101, mogli identifikovati pacijenti (ne)podobni za amiloid-redukujuću terapiju. Stabilan nivo miR-101 ekspresije, ukazivao bi na efikasnost terapije, koristeći tako i u monitoringu njenog djelovanja.

Inverzni nivo ekspresije miR-101 kod pacijenata sa AB i CAC, takođe je razmatran u kontekstu potencijalne, inverzne molekularne povezanosti ovih bolesti. Ekspresija miR-101 je bila značajno povećana u AB, u poređenju sa CAC grupom. Smanjenje nivoa ekspresije miR-101 u oblasti kancera, najprije je demonstrirano na ćelijskim linijama karcinoma dojke (468), a potom dokazano i kod drugih tipova kancera (317,318,469,470,471). Smanjenje miR-101 ekspresije u CAC tkivu i kolorektalnim ćelijskim kulurama, takođe je identifikovano većim brojem istraživanja (314,321,473,474). Naime, dokazana je odbrambena uloga miR-101, kroz posredovanje u tumorskoj supresiji (313,314,318,474-479), a njeni antitumorski efekti u kontekstu CAC su nedvosmisleno pokazani, u različitim eksperimentalnim postavkama istraživanja (314,321,474,477,478). Stoga se smanjena ekspresija miR-101 u CAC dovodi u vezu sa smanjenom onkosupresijom, odnosno, smatra se faktorom koji doprinosi nastanku CAC. Stoga, ovi literaturni podaci jesu u skladu sa rezultatom smanjenja miR-101 kod pacijenata sa CAC, pronađenim u ovoj studiji.

Uzimajući u obzir odnos AB-CAC, dokazi o onkosupresivnom miR-101 efektu, zajedno sa rezultatima ovog istraživanja, koji ukazuju na trend povećanja miR-101 u ranoj AB, dovode nas do sljedećeg zaključka: dugi pretklinički AB stadijum koji može trajati nekoliko decenija (29,30,44,45,105,106), mogao bi stimulisati miR-101 up-regulaciju kao zaštitni mehanizam – u cilju smanjenja *APP* ekspresije, ali bi ova miRNK istovremeno manifestovala onkosupresivnu ulogu, potencijalno štiteći ove pojedince od razvoja kancera. Zapravo, ova hipoteza proizašla iz našeg istraživanja, pruža jedno od mogućih objašnjenja zašto oboljeli od AB imaju smanjen rizik za nastanak kancera. Kao korak dalje, pokušali smo da istražimo i da li bi miR-101 mogla ostvariti navedene suprotne efekte posredstvom targeta zajedničkog za obje bolesti i time još snažnije odbraniti svoju ulogu molekula-indikatora inverzne asocijacije AB i CAC. Analiza brojnih studija koje razmatraju miR-101 kao negativnog regulatora gena ciklooksigenaze-2 (eng. ciclooxigenase-2 - COX-2), sa proinflamatornim efektom u AB i onkogenim u CAC, jasno podržava predstavljenu hipotezu (317,318,473,480-490). Naime, dolazi se do istog zaključka - priroda AB-CAC odnosa je inverzna, ali izgleda da se ne bazira na inverznom nivou ekspresije miR-101. Umjesto toga, istovrsna, povećana regulacija miR-101 ima protektivni efekat u obje bolesti: povećana aktivnost COX-2 koja se detektuje upravo u ranoj AB u kontekstu neuroinflamatornog procesa, mogla bi biti uzrok povećane miR-101 ekspresije - kako je pronađeno u našoj studiji, sa ciljem da svojom negativnom regulacijom suprimira inflamaciju. Istovremeno bi došlo i do inhibicije onkogenog djelovanja COX-2. Drugim riječima, očekuje se da će povećana ekspresija miR-101 uzrokovana AB, zaštititi ove pojedince od kancera, negativnom regulacijom COX-2, zajedničkog targeta koji posreduje i u neuroinflamaciji i u kancerogenezi. Konačno, bez obzira da li se razmatraju efekti simultanog uticaja miR-101 na različite ili na target zajednički za obje bolesti, dolazimo do zaključka da se jasno inverzan odnos AB i CAC, reflektuje kroz izmijenjenu ekspresiju miR-101 indukovanu jednom od ovih bolesti. **Dakle, rezultati prikazanog istraživanja potvrđuju treću hipotezu.** Osim toga, istraživanje inverzne korelacije između AD i CAC, dovelo nas je do predstavljanog i potencijalno novog objašnjenja uloge miR-101 u AB, koja do sada nije pokazana. Stoga **konačno, ovo istraživanje je rezultiralo i uspostavljanjem nove naučne hipoteze.**

Zaključak: Među pet istraživanih miRNK molekula, ovom studijom su identifikovana čak tri potencijalna biomarkera AB. Dvije miRNK bi mogle biti od velikog značaja za stratifikaciju opšte populacije prema riziku za obolijevanje od AB. Neočekivano, identifikovana je i jedna miRNK koja bi potencijalno mogla da posluži u procesu dalje stratifikacije onih pacijenata sa već definisanom AB, u kontekstu primjene najnovije terapije koja modifikuje bolest. Jednostavnost, pristupačnost i neinvazivnost metode detekcije miRNK, predstavljaju dodatnu vrijednost, povećavajući mogućnost njihove buduće primjene u oblasti AB. Na kraju, rezultati prikazanog istraživanja nude molekularnu potvrdu, odnosno objašnjenje inverzne korelacije incidenci AB i CAC.

Konkretnije, ovim istraživanjem je otkriveno da određeni broj pojedinaca sa kognitivnim deficitom u crnogorskoj populaciji, nije prepoznat. Stoga bi neuropsihološke skrining testove trebalo rutinski primjenjivati kod starije populacije u Crnoj Gori, nezavisno od žalbi na kognitivne funkcije. Povećana ekspresija miR-146a and miR-155, mogla bi predstavljati neinvazivni cirkulišući biomarker, za detekciju pojedinaca sa kognitivnim deficitom, koji su u riziku za AB. Zajedno sa neuropsihološkim skriningom, ovi molekularni markeri bi mogli postati rutinska, neinvazivna sredstva za skrining opšte populacije u kontekstu AB.

MiR-101 negativno reguliše ekspresiju *APP* gena i APP metabolizam, stoga je njena smanjena regulacija koja karakteriše AB, shvaćena kao faktor koji doprinosi nastanku bolesti. Međutim, trend povećanja nivoa ekspresije miR-101 u EAD, kao i njeno opadanje sa progresijom bolesti otkriveno u ovoj studiji, sugeriše da bi miR-101 mogla djelovati u okviru mehanizma negativne povratne sprege, koji je u vezi sa APP metabolizmom. Uzimajući u obzir da miR-101 potencijalno reflektuje progresiju akumulacije amiloida, mogla bi služiti i u monitoringu efekata amiloid-redukujuće terapije, indikovane u EAD populaciji. Uzimajući u obzir onkosupresivnu ulogu miR-101, njena potencijalno povećana ekspresija u dugotrajnoj pretkliničkoj i ranoj fazi AB, mogla bi zaštititi AB pacijente od kancera. Zapravo, simultana negativna regulacija onkogena i *APP* gena, kroz povećanu regulaciju miR-101, kao tačke preklapanja različitih signalnih puteva, mogla bi objasniti inverzne incidence AB i CAC.

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BIOGRAFIJA

Isidora Rovčanin Dragović

Rođena je 1987. u Mojkovcu, gdje je završila osnovnu školu, a potom gimnaziju u Podgorici. Diplomirala je na Medicinskom fakultetu u Novom Sadu 2012. sa prosječnom ocjenom 9,23 i odbranila diplomski -istraživački rad sa ocjenom 10. Na Medicinskom fakultetu u Podgorici je 2013. upisala Doktorske studije i položila ispite sa prosječnom ocjenom 10.

Radila je kao ljekar Klinike za neurologiju Kliničkog centra Crne Gore, 2015 16. Specijalističke studije neurologije na Medicinskom fakultetu u Beogradu je upisala 2017. Sertifikovana je za neuropsihološku procjenu 2019. Zaposlena je na Katedri za fiziologiju Medicinskog fakulteta u Podgorici, gdje sprovodi praktičnu nastavu i naučno-istraživački rad.

U okviru Polaznog istraživanja je ispitivala uticaj magnezijuma na periferni nerv animalnog eksperimentalnog modela. Učestvovala je u međunarodnim projektima tokom kojih se edukovala iz oblasti biohemijskih i molekularno-bioloških tehnika, kao i za rad na ćelijskoj kulturi, u Institutu za istraživanje kancera i starenja u Nici i u Institutu za biomembrane i bioenergetiku u Bariju. U okviru nacionalnog projekta Medicinskog fakulteta, sprovela je translacionu neuronaučnu studiju za doktorsku disertaciju, kroz istraživanje uloga miRNK molekula u Alchajmerovoj bolesti. Samostalno je koncipirala još jedno istraživanje sa fokusom na Alchajmerovu bolest, čija je realizacija u toku.

Autor je i koautor 10 konferencijskih radova i 4 internacionalne žurnalske publikacije, kao i ad hoc recenzent u međunarodnim časopisima. Jedan je od urednika u vodećem međunarodnom časopisu – „Journal of Alzheimer's Disease".

Nagrade: diplome „Luča" za osnovno i srednje obrazovanje; najbolji istraživački rad na Internacionalnom kongresu studenata medicine u Novom Sadu, 2012; nagrada Ministarstva prosvjete Crne Gore za ostvareni prosjek 10 na Doktorskim studijama; najbolji prezentovan rad u oblasti Alchajmerove bolesti i demencija na Svjetskoj neurološkoj konferenciji o kontroverzama u neurologiji, 2022.

Govori engleski i njemački, služi se italijanskim i ruskim jezikom.

Majka je dvoje djece.

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