HOW COMMON IN POLISH POPULATION ARE GENOTYPES OF APOLIPOPROTEIN E WHICH PREDISPOSE TO ALZHEIMER'S DISEASE DEVELOPMENT?

JAK CZĘSTE W POPULACJI POLSKIEJ SĄ GENOTYPY APOLIPOROTEINY E, KTÓRE PREDYSPOŃUJĄ DO ROZWOJU CHOROBY ALZHEIMERA?

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ABSTRACT
Introduction
Apolipoprotein E (ApoE) is a glycoprotein secreted mainly by hepatocytes. It’s involved in cell proliferation and cholesterol transport. ApoE is occurring primarily in 3 significant variants ε2, ε3 and ε4. It is coded by two genes what means the combination of two polymorphic alleles determines its genotype. ApoE-ε3/ε4 and -ε4/ε4 increase the risk of Alzheimer’s disease (AD) development several times compared to the general population. Early prevention can significantly slow down AD development and shorten its course.

Aim
The aim of this study was to check the distribution of ApoE gene among population from South Poland and compared results with previous study indicating allelic discrimination among random patients in research group.

Material and methods
In general we carried out genotyping polymorphism of ApoE. The process consisted of: 1. Collecting blood samples from patients, 2. Isolating DNA, 3. Preparing concentrations of DNA and checking it with spectrophotometer, 4. Allelic discrimination with fluorescent – labelled probes, 5. Genotyping of the ApoE polymorphism using Roche Lightcycler96 device. Study group consisted 830 subjects. 1660 determinations has been conducted.

Results
After expanding the research group by 81 patients it is shown that amount of carriers ApoE-ε2/ε4, ApoE-ε3/ε4 and ApoE-ε4/ε4 increases. The most numerous genotypes in research group are ApoE-ε2/ε2 and ApoE-ε3/ε3.

Conclusions
An upward trend in the relative numbers of carriers of genotype associated with a higher risk of developing AD has been noticed with the extension of research. Further research should be performed and the research group extended to check if observed tendency will contended with expanding group.

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**Keywords:** dementia, predisposing gen, genetic research

**STRESZCZENIE**

**Wstęp**

Apolipoproteina E (ApoE) jest glikoproteiną wydzielaną głównie przez wątrobę. Uczestniczy w proliferacji komórek i transporcie cholesterolu. Występuje przede wszystkim w 3 istotnych wariantach ε2, ε3 i ε4. Kodowana jest przez dwa geny, w związku z tym jej genotyp stanowi kombinację tych polimorficznych alleli. ApoE ε3/ε4 oraz ε4/ε4 zwiększa kilkukrotnie ryzyko zachorowania na chorobę Alzheimera (AD) w stosunku do populacji ogólnej. Badania wskazują, że stosując odpowiednio wcześnie prewencje, można znacznie spowolnić jej rozwój i złagodzić przebieg AD.

**Cel**

Celem badanie było sprawdzenie rozkładu genotypów genu ApoE w populacji Południowej Polski i porównanie jej do wyników poprzednich badań określających dyskryminację alleliczną u pacjentów losowo dobranych do grupy badawczej.

**Materiał i metody**

Przeprowadzono genotypowanie polimorfizmu ApoE. Przeprowadzenie materiału obejmowało: 1. Pobranie próbek krwi od pacjentów, 2. Isolacje DNA, 3. Przygotowanie stężeń DNA i ich kontrolę przy pomocy spektrofotometru, 4. Dyskryminację alleliczną z użyciem znakowanych fluorescencyjnie sond, 5. Genotypowanie polimorfizmu ApoE aparatem Roche Lightcycler96. Grupa składała się z 830 pacjentów (losowo dobrani pacjenci). Wykonano 1660 oznaczeń.

**Wyniki**

Po rozszerzeniu grupy o 81 pacjentów wzrosła ilość pacjentów z genotypami ApoE-ε2/ε4, ApoE-ε3/ε4 oraz ApoE-ε4/ε4. Najliczniejszymi genotypami były ApoE-ε2/ε2 i ApoE-ε3/ε3.

**Wnioski**

Zauważono trend wzrostowy we względnej liczbie nosicieli genotypów powiązanych ze zwiększonym ryzykiem zachorowania na AD po rozszerzeniu grupy badawczej. Dalsze badania powinny zostać przeprowadzone, a grupa badawcza poszerzona w celu sprawdzenia czy zauważona tendencja jest utrzymana przy zwiększaniu grupy.

**Słowa kluczowe:** demencja, gen predysponujący, badanie genetyczne

**Introduction**

1. **Characteristics of Apolipoprotein E – functions and distribution**

Apolipoprotein E (ApoE) is a glycoprotein consisting of 299 amino acids, with a mass of 34 kDa. Its gene is located on the long arm of chromosome 19. It is synthesized by hepatocytes as well as by neuronal cells (astrocytes), adipocytes, smooth muscle cells and macrophages (Lee et al. 2010; Tudorache et al. 2017). ApoE is involved in many processes in the human body. It participates in lipid metabolism, cell proliferation, lymphocyte activation as well as pathogenesis of Alzheimer’s disease (AD) (Tudorache et al. 2017). It plays an important role in the transport of cholesterol. It facilitates removal of triglyceride-rich lipoproteins from plasma (Mahley et al. 2009), which is important in prevention on atherosclerosis.
2. ApoE isoforms
There are three important variants of ApoE, among which all ApoE isoforms are expressed by polymorphic alleles ε2, ε3 and ε4. The difference between them is a single amino acid. In ApoE-ε2 isoform both cysteines are in positions 112 and 158. In ApoE-ε3 cysteine is in position 112, while arginine is in 158. In contrast in ApoE-ε4 arginine is present in both locations. The difference between them is Single Nucleotide Polymorphism (SNP) in two locations, referred to by the numbers rs429358 and rs7412. In addition ApoE is coded by two genes, so the combination of two variants determines its genotype, as shown in Table 1.

| ApoE ε2/ε4 | ApoE ε2/ε2 | ApoE ε2/ε3 | ApoE ε3/ε3 | ApoE ε3/ε4 | ApoE ε4/ε4 |
|------------|------------|------------|------------|------------|------------|
| rs429358   | (C:T)      | (T:T)      | (T:T)      | (C:T)      | (C:T)      |
| rs7412     | (C:T)      | (T:T)      | (C:T)      | (C:C)      | (C:C)      |

A second process implicated in AD development is neurofibrillary degeneration. It involves hyperphosphorylated tau protein, which lost its original ability of building the nerve cell skeleton.

Both processes result in decrease in the number of cortical neurons. The areas affected most commonly are olfactory bulb and the medial part of the temporal lobes, but parietal and frontal lobes may also be involved.

These degenerative changes lead to abnormal neurotransmission: mainly cholinergic, but a reduction in the amount of noradrenaline, serotonin and dopamine is also observed. Therefore inter- and intraneuronal communication is disturbed. It consequently gives rise to clinical symptoms characteristic of AD, such as memory dysfunction, especially short-term concerning current events, cognitive decline and attention deficit.

It is also worth noting that pathogenesis of AD involves processes such as: increased levels of oxidative stress, formation of pathological ion channels and chronic inflammatory response. The pathogenesis of AD is multifactorial, in which aging processes overlap with environmental factors and genetic predisposition. (Kozubski et al. 2014; Podemski 2019).

3. Aetiology and pathogenesis of Alzheimer’s disease
The main hypothesis of AD pathogenesis is the theory of ‘amyloid cascade’. In this theory primary point is the accumulation of proteolysis-resistant and insoluble amyloid β (Aβ) proteins in the brain. Aβ is a fragment of endothelial amyloid precursor protein (APP). APP is susceptible to the enzymatic degradation by secretase. The initial step of amyloidogenesis process is fragmentation of amyloid molecules by β and γ secretases. This leads to accumulation of Aβ including toxic oligomeric forms.

4. Epidemiological data on Alzheimer’s disease
The proportion of population aged 65 and above in the western European countries is already high and will increase. This represents a group at high risk of developing cognitive dysfunction and dementia. AD is the most common of a progressive dementia in older adults. It affects 5–7% of people over 65. (Szczudlik 2016).

Worldwide the AD affects 25.6 million people and it is estimated that by 2050 this number will increase to 106.8 million, including
16.51 million in Europe. Researches confirm the upward trend, which predicts a 87% increase in Europe between 2010 and 2050 (Niu, 2017). It is estimated that in Poland there are between 300,000 and 500,000 individuals with dementia. As a cause of disability and death dementia is one of the major public health challenges in the elderly population (Szczudlik 2016).

It is important to mention that even late-onset AD begins most often in the middle age, decades before dementia symptoms appear. This implies that patients who present AD at age 85 had changes in the brain as early as in their 50’s. Those who present at age less than 65 had abnormalities as early as 35. This phase is called prodromal and offers opportunities for early intervention and preventing changes. (Shokri-Kojori et al. 2018).

5. Apolipoprotein E and Alzheimer’s disease
As it was mentioned earlier, one of the causes of AD is accumulation of extracellular Aβ. Studies prove that ApoE affects the formation of β-amyloid. ApoE-ε4 isoform causes an increase amount of Aβ. If person is homozygous and has the ApoE-ε4/ε4 genotype, the risk of developing Alzheimer’s disease is several times higher than in the general population. However having the genotype ε2/ε2 indicates a protective effect on the development of AD (Huynh et al. 2017; Podemski 2019).

PET imaging of the brain confirms this relationship. This imaging was performed to establish correlation between the adverse genotype and Aβ fibrillar load. The presence of ApoE-ε4 variant has been shown to be closely related to β-amyloid changes in the frontal, temporal and parietal cortex. This connection was stronger in homozygotes compared to heterozygotes (Reiman et al. 2009).

Association was proven by metanalysis of 139 studies from various regions of the world. It has also been shown that allele ε4 is present in more than 50 percent of patients with AD, as opposed to 15% in healthy subjects. Another conclusion from these studies was that the frequency of the ApoE-ε4/ε4 genotype is also dependant on the region and ethnicity (Ward et al. 2012).

Cacciaglia and co-workers conducted a study on 533 people using volumetric MRI scans – the gray matter network (GMn) method. This demonstrated structural brain abnormalities in AD, specifically decrease in gray matter volume. The effect of allele ε4 on GMn was confirmed. These studies indicated that carriers of unfavorable genotype with asymptomatic AD have morphometric changes in their brains, even when they are not associated with pathologies of amyloid structure (Cacciaglia et al. 2020).

Inflammation is an important factor of the development of AD. Studies have shown that ApoE is one of the risk factors of neuritis. ApoE isoforms affect the inflammatory processes in microglia and astrocytes in varying degrees. ApoE-ε4 exhibits the weakest anti-inflammatory effect. Other isoforms are more effective in reducing the amount of proinflammatory cytokines in brain. It has been shown that carrying an unfavorable genotype is associated with a stronger inflammatory response, not only local but also systemic (Lynch et al. 2003). It is worth mentioning that blockade of the inflammatory signaling increases the expression of ApoE in microglia. This is most likely caused by a negative feedback loop. That means ApoE inhibits inflammation and inflammation inhibits ApoE levels (Pocivavsek et al. 2009). In addition, the reduced anti-inflammatory effect of ApoE-ε4 may play an important role in various neurological diseases in which neuritis and glial activation are observed, like sclerosis, head injuries as well as increased probability of developing the AD (Lynch et al. 2003).

It is estimated that one-third of Alzheimer's cases can be potentially prevented by modification of risk factors. For this reason genetic testing is extremely important. In studies it was determined how influence the application of prevention on ApoE-ε4 carriers. Diet, alcohol consumption, smoking and physical activity were taken into
consideration. Physical activity is one of the main factors preventing the development of AD. It was also noted that this connection is stronger among carriers of allele ε4 (Kivipelto et al. 2008). In other study it was shown that in comparison to physically active carriers of ApoE-ε4/ε4, people with an unfavorable genotype and low physical activity had a higher level of Aβ in the brain. In addition among group of people who often play sports, Aβ levels in brain were similar regardless of genotype (Head et al. 2013).

Studies indicate that three servings of wine per day were associated with a lower risk of AD, but only for people who are not carriers of the unfavorable genotype. Any amount of alcohol for ApoE-ε4 carriers increases the risk of developing AD (Head et al. 2013). Studies also showed that carriers of allele ε4 who smoked cigarettes had increased Aβ deposition in brain compared to non-smoker carriers (Cremonte et al. 2014). According to researches, adherence to the Mediterranean diet is more important in prevention AD for people without allele ε4 (Mosconi et al. 2014). It was proven that homozygotes ApoE-ε4/ε4 with high vitamin D concentration had better cognitive functions in comparison to ApoE-ε4/ε4 carriers whose concentration was lower (Maddock et al. 2015).

Aim
The goal of this study was to establish the distribution of ApoE variant among Polish population from Upper Silesia and to compare current results with previous published studies (Poręba et al. 2019).

Materials and methods
All patients signed the consent to be part of the project. The process of DNA isolation and genotyping was performed in the laboratory which is a part of to the Department of Internal Medicine, Diabetology and Nephrology in Zabrze.

The initial step was collection of the blood from the subjects. The samples were stored at -70°C until the collection was completed. Isolation the DNA material followed. In the next step desired concentration of DNA, which was 15 ng/μl was prepared. Lastly the concentrations were confirmed with a spectrophotometer.

After that the allelic discrimination was performed using fluorescently labeled probes and LightCycler96 from Roche. The alleles were labeled as C in VIC and as T in FAM.

1498 initial determinations were carried out utilizing DNA from 749 patients from Upper Silesia. After statistical analysis was completed, 81 new patients were recruited to assess if sample size change the results. Laboratory process described above was utilized for this new analysis.

All of the 830 study subjects resided in Southern Poland. 460 were women and 370 were men. Total 1660 samples have been made.

Results
After inclusion of 81 new patients, we showed the following significant increase was observed: carriers of ApoE-ε2/ε4 increase from 20 to 33 people, ApoE-ε3/ε4 from 79 to 138 and ApoE-ε4/ε4 from 36 to 43 (Table 2). It is notable that ApoE-ε4, associated with AD adverse effects, was presented in all three groups.

ApoE-ε2/ε2 (26% of participants) and ApoE-ε3/ε3 (26% of participants) remined the most common genotypes in our research group, but their relative percentage has decreased. Significant increase in the amount of carriers of ApoE-ε3/ε4 was noticed. Above described trends are illustrated in Figure 1 (Figure 1).

When analyzing the distribution of genotypes in research group by gender (Table 3, Figure 2), statistically significant predominance of women over men is observed. 6% of women vs 5% of men was observed in the group of ApoE-ε4/ε4 carriers. Similarly, in the ApoE-ε3/ε4 subset can be noticed 17% of participating women comparing to 16% of men. Also in the ApoE-ε3/ε4 group the number of women increased from 48 in the primary group to 80 in the extended group
Table 2. Distribution of ApoE genotypes in research group- amount of carriers and percent in group.

| Genotype of ApoE | Primary group | | Extended group | |
|------------------|---------------|---------------|---------------|---------------|
|                  | Amount | Percent | Amount | Percent | |
| ApoE-ε/ε/ε       | 40     | 5%      | 40     | 5%      | |
| ApoE-ε/ε/ε/ε     | 69     | 9%      | 69     | 8%      | |
| ApoE-ε/ε/ε/ε     | 8      | 1%      | 8      | 1%      | |
| ApoE-ε/ε/ε/ε     | 215    | 29%     | 216    | 26%     | |
| ApoE-ε/ε/ε/ε     | 70     | 9%      | 70     | 8%      | |
| ApoE-ε/ε/ε/ε     | 20     | 3%      | 33     | 4%      | |
| ApoE-ε/ε/ε/ε     | 212    | 28%     | 213    | 26%     | |
| ApoE-ε/ε/ε/ε     | 79     | 11%     | 138    | 17%     | |
| ApoE-ε/ε/ε/ε     | 36     | 5%      | 43     | 5%      | |

Figure 1. Distribution of ApoE genotypes in research group- comparison of the extended group to the primary group.

Table 3. Distribution of ApoE genotypes in reference to gender in research group- amount of carriers and percent in group.

| Genotype of ApoE | Primary group | | Extended group | |
|------------------|---------------|---------------|---------------|---------------|
|                  | Women | Men | Women | Men | |
| ApoE-ε/ε/ε       | 21    | 19  | 21    | 19  | |
| ApoE-ε/ε/ε/ε     | 38    | 31  | 38    | 31  | |
| ApoE-ε/ε/ε/ε     | 6     | 2   | 6     | 2   | |
| ApoE-ε/ε/ε/ε     | 122   | 93  | 123   | 93  | |
| ApoE-ε/ε/ε/ε     | 35    | 35  | 35    | 35  | |
| ApoE-ε/ε/ε/ε     | 13    | 19  | 19    | 14  | |
| ApoE-ε/ε/ε/ε     | 112   | 100 | 112   | 101 | |
| ApoE-ε/ε/ε/ε     | 48    | 31  | 80    | 58  | |
| ApoE-ε/ε/ε/ε     | 21    | 15  | 26    | 17  |
and the amount of men almost doubled from 31 to 58.

**Discussion**

AD is the most common cause of dementia syndrome, accounting for up to 60–70% of cases. Results from epidemiological studies from developed countries indicate that in 2016 47.5 million people worldwide were suffering from dementia syndrome. Among them up to 33.3 million were due to AD (Prince et al. 2016).

WHO estimates that in 2030 the total number of people affected by dementia will reach 75.6 million, and this number will rise to 135.5 million by 2050. It follows that the number of people suffering from AD will triple by 2050 (WHO 2017).

Both medical and social problems associated with AD do not only affect people in whose the symptoms had accrued but also their families and carers.

The gradual worsening of the patient’s condition in terms of cognitive abilities and their progressive loss of psychological and physical independence results in the need for continuous, 24 hours per day care (Miklis et al. 2016).

‘Alzheimer Europe’ research has shown that in Poland almost all people suffering from AD stay at home and their caregivers are mainly spouses or their children (43%). The vast majority are women (70%). Only 3% of patients are placed in special facilities.

The required care for the sick typically lasts around 8–12 years until the death of affected patients. Dementia is an overwhelming problem not only for the patients but also for his carers and family. Physical, emotional and financial pressures are a source of tremendous stress for everyone involved.

Several characteristics of people suffering from dementia such as cognitive impairment, behavioral changes, symptoms of depression, physical disability, as well as financial difficulties, social isolation and lack of social support make them vulnerable to potential abuse (Melchiorre et al. 2017).

Given the high number of subjects in our experiment the results can be extrapolated to general population. This can be used to predict the scale of the problem locally, to prepare the health care system and to educate the public.

As a result the adequate allocation of the budget for the prevention as well as treatment...
and care for patients suffering from this disease could be established. Addressing currently observed shortage of high quality long term care facilities would be of particular importance.

As mentioned previously, the cases of dementia including AD are increasing in number.

The relative contribution of genetic versus environmental factors in pathogenesis of dementia should be studied. For example individuals without high risk genotypes could be targeted (specifically carriers of negatively predisposing ApoE-ε4 allele) to determine the influence of the environment. In particular, identification of currently preventable causes is important.

Population screening for ApoE-ε4 may identify high risk individuals in the population and allow for their close monitoring and early intervention, starting with lifestyle modification and early treatment. Such measures may be efficacious given the fact that the first changes in the brain of AD patients appear as early as 10 to 25 years before the onset of clinical symptoms.

Based on available data the number of existing specialized care facilities does not meet the demand. Also the caregivers/family knowledge about AD is lacking (Miklis et al., 2008). By identifying the at risk patients early we create opportunity for educating the patient and his relatives is created. This early information may help the affected individuals to plan for the future and to take preventative measures. As a result the onset of symptoms and resulting social isolation could be delayed.

**Conclusions**

Increasing trend in the relative number of carriers of genotype associated with a higher risk of developing AD has been demonstrated.

In connection with the above, the at risk population in Poland is likely higher than previously reported.

When analysing the data with respect to gender, a relative predominance of women carriers of ApoE-ε3/ε4 and ApoE-ε4/ε4 was again seen in extended group.

Further research should be performed on even larger cohort for more complete estimate of the discriminatory alleles gen of ApoE genotypes in the population of Poland.

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