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Altered granulocyte count and erythrocyte measures in middle-aged, healthy carriers of *APOE* and *PICALM* risk genes for Alzheimer's disease

Patrycja Dzianok, Ewa Kublik*

Laboratory of Emotions Neurobiology, Nencki Institute of Experimental Biology PAS, Warsaw, Poland,
** Email: e.kublik@nencki.edu.pl*

APOE-ε4 genotype (apolipoprotein E, epsilon 4) is the strongest genetic risk factor for Alzheimer's disease (AD). Despite years of research, it is still not known how it contributes to dementia development. *APOE* has been implicated in many AD pathology mechanisms, like Aβ clearance, brain metabolism, changes within microglia and other glial functions and inflammatory processes. In fact, immunological/inflammatory processes are recently discussed as an important factor in Alzheimer's development and granulocyte profiles changes are reported in patients. However, the exact link between immune system and risk-genes is unknown. In particular, it is not known whether and how they interact throughout the lifetime, before the disease onset. The aim of the study was to investigate the relationship between granulocyte count and the *APOE/PICALM* genes in healthy individuals with an increased genetic risk of AD. An exploratory analysis regarding other blood cells was also conducted. Blood samples were collected from 77 healthy middle-aged (50–63 years old) participants, who were also asked to complete a health and life-style questionnaires. Groups with different AD risk-genes were compared. Differences in granulocyte profiles were found in healthy carriers of AD risk-genes who had slightly elevated eosinophil levels as compared to non-risk carriers. An exploratory analysis showed some alteration in mean corpuscular hemoglobin content and concentration (MCH/MCHC) levels between risk-carriers subgroups and non-risk carriers. No other differences in blood count or lipoprotein profile were found between healthy *APOE/PICALM* risk-carriers and non-risk carriers. Longitudinal studies will reveal if and how those changes contribute to the development of AD pathology.

Key words: Alzheimer's disease, eosinophils, granulocytes, *APOE, PICALM,* inflammation, peripheral immune cells

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder with an early, genetic form (5% of all AD cases, EOAD) and a late-onset form (95% of all cases, LOAD) (Barber, 2012), with unknown, complex etiology. These two distinct forms of the disease differ in their age of onset and underlying genetic factors. EOAD affects individuals who are younger than 65 years of age, in contrast to the LOAD that affect individuals who are 65 years or older (Alzheimer's Association, 2022). EOAD accounts for only a small percentage of all AD cases, but it is often associated with strong genetic factors. Mutations in three genes have been identified as causative for EOAD: the amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2) (Barber, 2012). Clinically, LOAD and EOAD present with similar symptoms, including memory loss, cognitive decline, and behavioral changes, but EOAD develops more rapidly. LOAD develops slowly, leading to increasing impairment of cognitive and social functions until the patient becomes completely incapacitated. It is associated with many risk factors, including older age, family history of dementia, and genetics, as well as modifiable risk factors such as diet, physical activity, social activity, mental activity, and smoking (Alzheimer's Association, 2022). Among the risk-genes, the best understood and that with the greatest impact on LOAD is a gene of

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apolipoprotein E (*APOE*) (Saunders et al., 1993; Liu et al., 2013).

Three forms of *APOE* exist: ε2, ε3 and ε4, with one of each variant inherited from each parent, resulting in six possible genotypes i.e., ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4 and ε4/ε4. *APOE*-ε3 is believed to be neutral, whilst *APOE*-ε2 has been reported as neutral or protective in some studies (Saunders et al., 1993; Corder et al., 1994). Possession of one ε4 allele increases the risk of AD three-fold, with two copies increasing risk by 8-12 times (Michaelson, 2014; Alzheimer's Association, 2022). More than half of AD patients are *APOE*-ε4 carriers, although some *APOE*-ε4 carriers will never develop the disease (Michaelson, 2014; Alzheimer's Association, 2022). *APOE* is related to cognitive decline, dementia, and cardiovascular disease risk (Bretsky et al., 2003; Liu et al., 2013). Despite almost 30 years of *APOE* research (first publication in 1993, Saunders et al., 1993), the core mechanism through which it contributes to dementia development is not yet fully understood. Lipid transport and cholesterol carrying are the primary roles of the ApoE protein, as it binds to cholesterol and lipoprotein molecules, as well as Aβ and tau proteins (Potter and Wisniewski, 2012; Liu et al., 2013). ApoE also enhances proteolytic degradation and clearance of Aβ, with the ε4 isoform being least effective in this respect (Potter and Wisniewski, 2012). These mechanisms partially explain its association with the formation of tau and Aβ deposits, and thus its contribution to AD. It was also shown that fibrillar Aβ load is different in cognitively unimpaired individuals with *APOE* risk (Snellman et al., 2023).

Phosphatidylinositol binding clathrin assembly protein gene (*PICALM*) is another risk-factor (confirmed by genome-wide association studies (Harold et al., 2009) related to amyloid pathology. *PICALM* is believed to be involved in modulating Aβ production and its transport and removal/cleaning, and may be related to tauopathy, synaptic dysfunction, and immune disorders (Xu et al., 2015). *PICALM* rs3851179 G/G alleles are believed to be the risky variants, with A/A being neutral or protective (Zeng et al., 2019). Mixed A/G variants are considered neutral in most research papers. Some research has shown a possible interaction of *APOE* and *PICALM* genotypes on LOAD development and impaired cognitive performance among healthy subjects and AD patients (Jun et al., 2010; Barral et al., 2012; Morgen et al., 2014). This included reduced memory functioning (Barral et al., 2012), brain atrophy, cognitive impairment (Morgen et al., 2014), and development of AD in general (Jun et al., 2010).

AD neuropathology is believed to start decades before the first symptoms of cognitive impairment (Blennow et al., 2015). The impact of risk factors on cognitive function and health of young and middle-aged asymptomatic, healthy individuals are studied, with the aim of identifying early LOAD biomarkers (Barral et al., 2012; Zhang et al., 2022). The involvement of inflammation at the onset and during the development of AD has been widely discussed (Kinney et al., 2018; Snellman et al., 2023). In the brain, the immune system is composed primarily of specialized glial cells and Aβ deposits are often surrounded by microglia and astrocytes (Dickson, 1997). Microglia and astrocytes produce most of the anti-inflammatory mediators and immune molecules in the brain; they are also the main source of ApoE. It was shown that both are activated in early AD (Britschgi and Wyss-Coray, 2007). Moreover, their functions are altered in ε4 carriers (Fernandez et al., 2019). Peripheral/chronic inflammation may also be involved, as there is emerging evidence that leukocytes, including granulocytes, may play an important role in AD pathophysiology (Stock et al., 2018; Järemo et al., 2013). While the blood-brain barrier (BBB) should stop peripheral cells from affecting the brain, it has been shown that some factors may increase the permeability of the BBB and therefore allow entry of peripheral cells to affect central nervous system functions (see Wang et al., 2019 for the review and description of the possible mechanism). In this regard, *APOE*-ε4 was shown to be related to BBB dysfunction (Montagne et al., 2020), and breakdown of the BBB seems to occur early in AD. Such breakdown by itself can lead to cognitive dysfunction independent of Aβ and tau accumulation (Nation et al., 2019). Granulocytes, including eosinophils, neutrophils and basophils, are the first cells of the immune system to come into contact with pathogens and bacteria. Although granulocytes protect against pathogens, their continued activity in the course of chronic (even subclinical) inflammation may cause tissue damage (Stock et al., 2018).

There is no cure for AD, available drugs are only able to slow down the progression of the neurodegeneration (Alzheimer's Association, 2022), and there is currently no way to stop or reverse progression of a disease, which is typically diagnosed at its late, symptomatic stages. Therefore, it is crucial to understand the background and mechanisms of AD development and to develop the earliest possible diagnosis and risk assessment strategies. Knowledge of the impact of risk genes on healthy individuals at different stages of life should be expanded. Since inflammatory processes affect our organisms throughout the whole life, their interaction with other AD risk factors should be investigated. Hence, this study focused on the distribution of granulocytes in individuals with a genetic risk of AD associated with the *APOE* and *PICALM* genes. An exploratory analysis regarding other blood cells was also conducted.

METHODS

A total of 77 middle-aged (50-63 years old) participants (Caucasian, Polish citizens) were tested using basic blood tests that were outsourced to a third-party, certified, medical laboratory facility. Blood samples were collected in the morning by a trained nurse. All participants gave informed written consent to participate in the study and received cash remuneration. The local bioethical committee approved the study (Bioethics Committee of the Nicolaus Copernicus University in Toruń functioning at Collegium Medicum in Bydgoszcz, Poland).

Only healthy participants were enrolled in the study (this requirement was written on the study information, which was signed by each participant): participants were not demented and in generally good health, which was confirmed by the psychometric and health questionnaires. All participants were literate and educated. The exclusion criteria included recent or ongoing infection, left-handedness, general excessive health problems, epilepsy, known mental illness or brain damage, chronic headaches, sleep disorders, skin diseases, metal objects/implants in the body and pregnancy. Due to their age, some of the participants were taking medications for various conditions, such as back pain, hormonal problems, hearing problems or other. However, all participants were well-functioning (including neuropsychologically), able-bodied and professionally active (or early retired in a few cases). Some of the exclusion criteria listed above were set because participants were also enrolled in a study that includes neuroimaging. The dataset from the neuroimaging study has been analyzed and will be publicly available in the near future as the Polish Electroencephalography, Alzheimer's Risk-genes, Lifestyle and Neuroimaging (PEARL-Neuro) Database, and there are plans to continuously extend the database in the future.

All participants filled in a subjective questionnaire related to sociodemographic AD risk factors (age, sex, smoking status etc.) and vascular risk factors (hypercholesterolemia, diabetes, hypertension). Alcohol use was assessed with a screening test, the Alcohol Use Disorders Identification Test (AUDIT, threshold of ≥8 points could indicate unhealthy alcohol usage). To calculate body mass index (BMI), participants were asked for their weight and height. Healthy weight is between 18.5-24.9 BMI, overweight ≥25.0, and obesity ≥30.0 BMI. Height, weight, and allergies, were assessed by the questionnaire and not objectively tested.

Participants for blood tests were chosen from a larger cohort (N=200) who underwent genotype screening by standard DNA Sanger sequencing technique (commissioned to the Genomed S.A., Poland). Alleles of the AD risk genes *APOE* (rs429358/rs7412) and *PICALM* (rs3851179) were determined. One participant with an ambiguous *APOE* ε2/ε4 genotype was excluded from the analysis, as ε2 is a rare, possibly protective allele, and ε4 is a risk factor. Data from a total of 76 subjects were analyzed. Experimental groups were defined by the different AD risk genes, but were otherwise balanced in regard to other physiological and life-style features.

The group of *APOE*-ε4 carriers was composed of 45 participants with or without an additional risk factor for the *PICALM* gene, and *APOE*-ε4 non-carriers were all characterized by the *PICALM* AA/AG neutral/beneficial genotype (Table 1). Firstly, the two main *APOE* groups were compared (*APOE*-ε4 risk-carriers, n=45; named 'R') *vs.* non-risk carriers (n=31; named 'N'). This general contrast would also allow for comparisons to other studies, typically not identifying *PICALM* subgroups. *APOE*-ε4 carrier status was classified as having at least one ε4 allele (it is worth noting that there were only two subjects in this group with a homozygous ε4/ε4 genotype). In the second stage, the '*APOE* risk' group was split into 'Single-risk' (*APOE*-ε4 carriers not accompanied by *PICALM* risky GG alleles, n=24) and 'Double-risk' (*APOE*-ε4 accompanied by *PICALM* GG alleles, n=21) subgroups. The groups of this comparison are abbreviated in the following paragraphs and figures as follows: 'N' non-risk group, with neutral/protective *APOE/PICALM* alleles, 'A+P+' 'double-risk' group with risky *APOE/PICALM* alleles and 'A+P-' with *APOE* risky alleles and *PICALM* neutral/protective alleles.

A complete blood count panel was performed, including hemoglobin (and mean corpuscular hemoglobin content and concentration, MCH, MCHC), hematocrit, erythrocytes, leukocytes and platelets etc.

Table 1. Allele distribution of *APOE* and *PICALM* risk genes carriers and non-risk carriers.

	APOE-ε4 carriers (A+; N=45)	*APOE*-ε4 non-carriers (A-; N=31)
PICALM GG carriers (P+; N=21)	21	0
PICALM AA/AG carriers (P-; N=55)	24	31 (N group)

'N' denominates non-risk carriers (A-P-) group.

A basic lipoprotein profile was performed to identify participants with potential hypercholesterolemia (Table 2). Herpes simplex virus (HSV) was also tested as it has been shown previously that HSV may be related to AD development (Itzhaki, 2021). All measurements taken are important for our longitudinal study, as we plan to investigate the same individuals in future to see if any of the middle age markers have an impact on later cognitive decline and/or the development of dementia.

Statistics

Statistical analyses were performed using Jeffreys's Amazing Statistics Program (JASP) (v.0.16.4) software. T-tests or Mann-Whitney U tests were used to compare granulocyte levels, other risk-factors and demographic information between two main *APOE* groups and an exploratory blood count differences (Mann-Whitney U tests were used when the assumption of normality was violated, as measured by the Shapiro-Wilk test of normality). Kruskal-Wallis test with Dunn's *post-hoc* analysis was used for comparison between three groups in regard to granulocyte levels (for each comparison at least one group validated the assumption of normality, as measured by the Shapiro-Wilk test of normality) and ANOVA or Kruskal-Wallis test were used for exploratory analysis of other blood cells (depending on the ANOVA assumptions being violated or not). Levene's test of equality of variances was used to test homogeneity, and all groups were valid with regards to granulocyte levels. For nominal variables (such as "sex", "possible alcohol problems", "smokers" etc.) chi-squared tests were performed. Significance of data was defined as follows: a p-value ≤ 0.05 was considered significant, and p-value > 0.05 and ≤ 0.09 was considered as a trend. All data are presented as mean (M) ± standard deviation (SD). Graphs were prepared with in-home Python (3.9.7) scripts, with use of the Seaborn package (Waskom, 2021) and matplotlib (Hunter, 2007).

Table 2. Demographic characteristics of the study groups.

Feature	Non-risk [N] (N=31)	Risk [R] (N=45)	N *vs.* R p-value	Risk subgroup: single-risk [A+P-] (N=24)	Risk subgroup: double-risk [A+P+] (N=21)	N *vs.* A+P- *vs.* A+P+ p-value
Age [years; M±SD]	54.77±2.92	55.80±3.29	*p*=0.17	55.96±3.33	55.62±3.31	*p*=0.36
Age [years; range]	51-60	50-63	–	50-62	51-63	–
Sex [F/M]	15/16	23/22	*p*=0.82	13/11	10/11	*p*=0.88
Possible alcohol problems [N]	1 (3.23%)	5 (11.11%)	*p*=0.21	3 (12.50%)	2 (9.52%)	*p*=0.43
Smokers [N]	2 (6.45%)	7 (15.56%)	*p*=0.17	5 (20.83%)	2 (9.52%)	*p*=0.12
Former smokers [N]*	7 (22.58%)	13 (28.89%)	*p*=0.75	9 (37.50%)	4 (19.05%)	*p*=0.52
Diabetes [N]	0 (0.00%)	1 (2.22%)	*p*=0.40	0 (0.00%)	1 (4.76%)	*p*=0.27
Hypertension [N]	5 (16.13%)	12 (26.67%)	*p*=0.28	8 (33.33%)	4 (19.05%)	*p*=0.29
Total cholesterol [mg/dl; M±SD]	206.33±41.11	210.67±43.88	*p*=0.61	209.08±38.11	212.49±50.59	*p*=0.84
Triglyceride [mg/dl; M±SD]	127.11±54.44	144.52±64.92	*p*=0.33	156.91±60.19	130.37±69.64	*p*=0.18
Allergies [N]**	7 (22.58%)	9 (20.00%)	*p*=0.73	5 (20.83%)	4 (19.05%)	*p*=0.94
BMI [M±SD]	26.70±4.35	27.94±5.65	*p*=0.41	27.34±5.81	28.58±5.55	*p*=0.44
BMI: overweight [N]	12 (38.71%)	17 (37.79%)	*p*=0.93	8 (33.33%)	9 (42.86%)	*p*=0.80
BMI: obesity [N]	7 (22.58%)	14 (31.11%)	*p*=0.41	7 (29.17%)	7 (33.33%)	*p*=0.68
Family history of dementia [N parents – one]	8 (25.81%)	18 (40.00%)	*p*=0.20	11 (45.83%)	7 (33.33%)	*p*=0.30
Family history of dementia [N parents – both]	0 (0.00%)	2 (4.44%)	*p*=0.23	1 (4.17%)	1 (4.76%)	*p*=0.49

M=mean, SD=standard deviation. F=females, M=males. N=number of subjects. *Former smokers: 1 score missing in N group. **Allergies: missing 3 scores in R group (2 in A+P+, 1 A+P-) and 3 in N group. 'C *vs.* R *p*-value' shows the results of exploratory analysis, the main effect of the *APOE* gene (the difference between the risk group 'R' and the non-risk group 'N'). 'N *vs.* A+P- *vs.* A+P+ *p*-value' shows the results of exploratory analysis, additional effect of both genes (the difference between the three groups).

RESULTS

Characteristics of the participants

Demographic/health/other risk-factor characteristics of the participants are reported in Table 2. Data of all participants eligible for further analysis were included (n=76). The population was middle-aged (55.38 ± 3.16), with age ranging from 50 to 63 years (Table 2). The gender ratio was maintained in each subgroup, with approximately equal numbers of men and women enrolled in the study. The prevalence of possible additional risk-factors (estimated from the questionnaire or blood tests) such as smoking, diabetes, hypertension, cholesterol, triglyceride levels and overweight/obesity was approximately the same for all groups (Table 2). Only a small percentage of participants suffered from allergies (as stated in the general questionnaire), with a similar proportion in each group (Table 2). Among the allergens mentioned were food (such as celery and leek, etc.), drugs (salicylates, non-steroidal anti-inflammatory drugs, antibiotics, etc.), and environmental/outdoor allergens (grass and tree pollen, artemisia etc.). Similarly, the percentage of participants with a family history of dementia (one or both parents affected) was balanced between the groups. There were no significant differences between the groups in terms of the demographic/health/other risk factors stated in Table 2.

Elevated eosinophils

Granulocyte counts were within the normal range (for laboratory norms, see supplementary Table S1) for the majority of participants (Table 3). Very few participants had slightly elevated or lowered scores, with no clear differences between the main groups.

The R group was characterized by higher eosinophil levels (U (N_R=45, N_N=31)=498.5, p<0.05, Fig. 1A, Table 4), showing main effects of *APOE* genotype. Mean levels of basophils and neutrophils were not significantly different between group N and group R (Fig. 1A). A trend level effect was seen for eosinophils count between the three analyzed subgroups (Table 5), possibly indicating an association between *APOE* and *PICALM* genes. Pairwise *post-hoc* Dunn test showed significant differences between A+P+ and N groups (p-value uncorrected; and a trend level for multiple-comparison adjusted p-value) for eosinophils counts, and a weak trend effect between A+P+ and A+P- groups for basophil count (uncorrected p-value; ns for adjusted p-value) (Table 5; Fig. 1B). No effects were found for neutrophil counts (Table 5; Fig. 1B).

Table 3. Number of subjects with elevated or lowered granulocyte levels.

	Non-risk [N] (N=31)	Risk [R] (N=45)	Risk subgroup: single-risk [A+P-] (N=24)	Risk subgroup: double-risk [A+P+] (N=21)
Above the norm level				
Eosinophils	0	1	0	1
Neutrophils	0	0	0	0
Basophils	1	1	0	1
Below the norm level				
Eosinophils	1	0	0	0
Neutrophils	2	3	1	2
Basophils	1	1	1	0

Table 4. Granulocyte tests results for the main risk groups and R subgroups.

Blood tests	N (N=31)	R (N=45)	R subgroups	
			[A+P-] (N=24)	[A+P+] (N=21)
Eosinophils [K/µl]	0.141±0.076	0.199±0.150	0.176±0.094	0.225±0.195
Basophils [K/µl]	0.047±0.024	0.050±0.023	0.043±0.019	0.058±0.025
Neutrophils[K/µl]	3.180±0.952	3.410±1.025	3.482±1.030	3.327±1.039

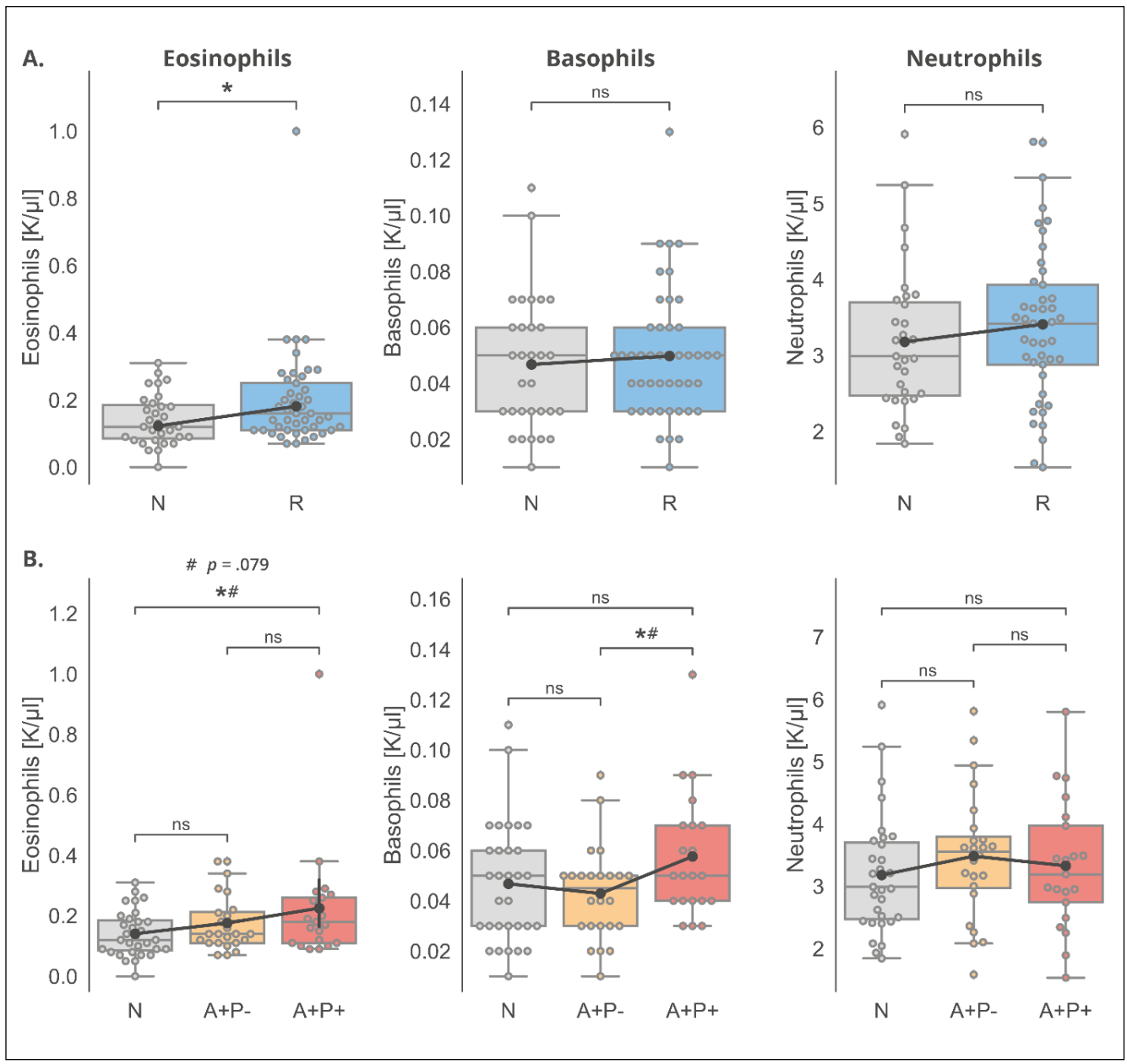

Fig. 1. Summary of granulocyte count. (A) Main effect of *APOE* genotype – risk carriers (R) *vs.* non-risk group (N) for eosinophils, basophils and neutrophils. The graph shows box plots with an additional representation of the distribution of individual data points denoted by grey dots. Black point plots on boxplots shows the mean change. The central horizontal bar shows the median, and lower and upper boundaries show the 25th and 75th percentiles. (B) Comparisons after dividing the R group to single-*APOE* risk (A+P-) and *APOE* & *PICALM* double-risk (A+P+). Similar distribution of results was used as described in point A | * p<0.05, *# significant result before the Holm-Bonferroni correction; ns – not significant. # Trend levels indicated by exact *p*-values.

Comparison of other blood cells profiles

There were no significant differences in other tests in the comparison of main two groups based on *APOE* variants only (R *vs.* N, Fig. S1). When considering the subgroups divided by *PICLAM* variant some differences were noted in red blood cell features. The A+P- group had higher hemoglobin content and concentration (MCH and MCHC) levels in comparison to the A+P+ and N groups (respectively: and) (Table 6). A trend difference between the groups was also noted for blood cell volume distribution (RDW-CV) (Table 6). Analysis revealed no differences between the groups with regard to other measurements described in Table 6. (Fig. S2).

Table 5. Statistical results for the differences between the three study groups.

Granulocytes type	Main statistics	*Post-hoc*		
		Groups	*p*-value uncorrected	*p*-value adjusted (Holm-Bonferroni test)
Eosinophils	H(2)=5.09, *p*=0.079 (#)	A+P+ *vs.* N	*p*<0.05 (*)	*p*=0.085 (#)
		A+P+ *vs.* A+P-	*p*=0.42	*p*=0.42
		N *vs.* A+P-	*p*=0.16	*p*=0.33
Basophils	H(2)=4.26, *p*=0.12	A+P+ *vs.* N	*p*=0.11	*p*=0.22
		A+P+ *vs.* A+P-	*p*<0.05 (*)	*p*=0.14
		N *vs.* A+P-	*p*=0.61	*p*=0.61
Neutrophils	H(2)=1.70, *p*=0.43	A+P+ *vs.* N	*p*=0.60	*p*=0.98
		A+P+ *vs.* A+P-	*p*=0.49	*p*=0.98
		N *vs.* A+P-	*p*=0.19	*p*=0.58

* marks significant effect; # marks trend level effect.

Table 6. Blood count test, lipoproteins profile and HSV virus tests results and exploratory comparison between the study groups.

Blood tests	N (N=31)	R (N=45)	N *vs.* R p-value	R subgroup: [A+P-] (N=24)	R subgroup: [A+P+] (N=21)	N *vs.* A+P- *vs.* A+P+ p-value
Leukocytes [K/µl]	5.95±1.40	6.34±1.54	*p*=0.27	6.28±1.37	6.41±1.74	*p*=0.52
Erythrocytes [K/µl]	4.93±0.45	4.84±0.43	*p*=0.39	4.80±0.41	4.89±0.46	*p*=0.54
Hemoglobin [K/µl]	14.67±1.55	14.65±1.26	*p*=0.96	14.77±1.32	14.51±1.21	*p*=0.82
Hematocrit [%]	44.58±3.70	44.02±3.31	*p*=0.49	44.00±3.15	44.05±3.57	*p*=0.79
MCV [fl]	90.55±4.05	91.36±4.06	*p*=0.46	92.04±3.78	90.57±4.32	*p*=0.34
MCH [pg]	29.77±1.75	30.33±1.51	*p*=0.31	30.88±1.39	29.71±1.42	p<0.01 *1
MCHC [g/dl]	32.83±1.30	33.17±0.85	*p*=0.16	33.48±0.91	32.82±0.62	p<0.05 *2
RDW-CV [%]	13.19±1.26	13.12±0.70	*p*=0.64	12.93±0.70	13.33±0.66	p=0.083 #3
Platelets [K/µl]	258.19±58.55	255.18±46.32	*p*=0.87	257.83±49.95	252.14±42.80	*p*=0.95
PDW [fl]	13.70±2.39	13.84±2.31	*p*=0.79	13.68±2.26	14.01±2.40	*p*=0.81
MPV [fl]	11.04±1.0	11.11±0.97	*p*=0.76	11.01±0.90	11.22±1.06	*p*=0.75
P-LCR [%]	33.74±8.27	34.27±8.12	*p*=0.78	33.49±7.64	35.16±8.75	*p*=0.77
Lymphocytes [K/µl]	2.02±0.60	2.10±0.72	*p*=0.46	2.00±0.51	2.21±0.91	*p*=0.75
Monocytes [K/µl]	0.54±0.14	0.56±0.14	*p*=0.45	0.56±0.14	0.57±0.15	*p*=0.74
HDL cholesterol [mg/dl]	57.60±18.02	56.58±17.10	*p*=0.94	53.86±15.97	59.70±18.18	*p*=0.55
No-HDL cholesterol [mg/dl]	148.73±39.51	154.10±38.53	*p*=0.56	155.22±35.94	152.80±42.17	*p*=0.83
LDL cholesterol [mg/dl]	123.31±35.68	125.18±36.30	*p*=0.69	123.84±32.40	126.72±41.08	*p*=0.91
HSV IgG positive [N]	27 [87.10%]	39 [86.67%]	*p*=0.96	20 (83.33%)	19 (90.48%)	*p*=0.78

All results show M±SD, M=mean, SD=standard deviation. N=number of subjects. MCV – mean corpuscular volume, MCH – mean corpuscular hemoglobin, MCHC – mean corpuscular hemoglobin concentration, RDW-CV – red blood cell distribution width, PDW – platelet distribution width, MPV – mean platelet volume, P-LCR – platelet-large cell ratio, HSV – herpes simplex virus. 'C *vs.* R p-value' shows the results of exploratory analysis, the main effect of the *APOE* gene (the difference between the risk group 'R' and the non-risk group 'N'). 'N *vs.* A+P- *vs.* A+P+ p-value' shows the results of exploratory analysis, additional effect of both genes (the difference between the three groups). *1 H(2)=9.27, *p*<0.01; A+P+ *vs.* N, *p*=0.44 (uncorrected), *p*=0.44 (Holm-Bonferroni correction); A+P+ *vs.* A+P-, *p*<0.01 (*p* uncorrected), *p*<0.05 (Holm-Bonferroni correction); N *vs.* A+P-, *p*<0.05 (*p* uncorrected), *p*<0.05 (Holm-Bonferroni correction) | *2 H(2)=6.94, *p*<0.05; A+P+ *vs.* N, *p*=0.58 (uncorrected), *p*=0.58 (Holm-Bonferroni correction); A+P+ *vs.* A+P-, *p*<0.05 (uncorrected), *p*<0.05 (Holm-Bonferroni correction); N *vs.* A+P-, *p*<0.05 (uncorrected), *p*=0.071 (Holm-Bonferroni correction); #3 H(2)=4.99, *p*=0.083, A+P+ *vs.* N, *p*=0.10 (uncorrected), *p*=0.21 (Holm-Bonferroni correction); A+P+ *vs.* A+P-, *p*<0.05 (*p* uncorrected), *p*=0.087 (Holm-Bonferroni correction); N *vs.* A+P-, *p*=0.48 (*p* uncorrected), *p*=0.48 (Holm-Bonferroni correction).

DISCUSSION

Standard blood measures were tested in healthy middle-aged participants with different burden of AD risk genes (*APOE, PICALM*). Participants also completed a questionnaire about lifestyle and health factors related to AD development. Most of the parameters were indistinguishable between control and risk groups, however the profile of granulocytes and erythrocyte hemoglobin content/concentration were different. Carriers of *APOE* and *PICALM* risk-genes for the late form of AD were characterized by slightly elevated (but still within a normal range) eosinophil levels and a small similar trend was seen for basophil count. The mean differences between the groups were small to moderate, which is not surprising in middle-aged and generally healthy individuals. This result corroborates recently published research (participants from Chinese Alzheimer's Biomarker and Lifestyle (CABLE) database, n=738, (Zhang et al., 2022) showing that peripheral eosinophil levels were higher in elderly, healthy individuals with AD biomarkers (Aβ42, Aβ42/p-tau) present in the CSF. Moreover, in Zhang's study, eosinophil levels increased with increasing levels of AD markers, and in our study, they were higher for double than single risk participants. The other blood test that was different between the groups in our study was the MCH and MCHC – effects not reported before in healthy risk carriers. They were previously shown to be decreased (Faux et al., 2014) or increased (Chen et al., 2017) in AD patients. The latter authors suggested that the increased MCH/MCHC among AD patients may be related to folic acid and cobalamin (i.e., vitamins B9 and B12) deficiency (Chen et al., 2017), which is also thought to contribute to AD pathogenesis (Faux et al., 2014). Additionally, folic acid supplementation was shown to reduce AD related inflammation and can therefore help with symptomatic treatment of AD (Chen et al. 2016). We have shown that MCH/MCHC levels were slightly elevated in the A+P- group, but were maintained at the control level in A+P+ group. GWAS studies showed before that *PICALM* rs3851179 AD risk/protectiveness was indicated in *APOE*-ε4 carries, as these two genes interact with each other (Harold et al., 2009). Additionally, it was suggested that the appropriate diet intervention is beneficial in both risk and protective *PICALM* alleles carries, improving their cognitive functioning (Martínez-Lapiscina et al., 2014). However, we do not have data regarding the levels of B9/B12 in our sample or other dietary information, therefore we cannot infer if it could explain the effect of the protective (P-) and risk (P+) *PICALM* alleles on the obtained results. It is easier to explain possible *PICALM* effects promoting AD, as it influences Aβ deposits and functioning of the synapses (Harold et al., 2009; Xu et al., 2015), with AD brains having fewer synapses (Terry et al., 1991). Some research has even shown that a lower number of synapses among AD patients correlates with cognitive deficits stronger than Aβ plaque and tau tangle burden (Terry et al., 1991; Colom-Cadena et al., 2020). Although synapse loss in AD may be an effect of amyloidosis, it can also be caused by tauopathy and inflammatory processes, so the mechanisms are linked (Colom-Cadena et al., 2020). In contrast, some cell-based studies have shown that risky *APOE*-ε4 variants correlated with enhanced synaptogenesis (Huang et al., 2019). This effect may be potentially beneficial in early life, but at later stages it may provoke enhanced synapse elimination (see commentary by Dzianok and Kublik, 2020 for the discussion of this effect).

As outlined in the introduction, inflammatory processed are believed to participate in AD development. Eosinophils are mostly implicated in respiratory diseases such as asthma, chronic obstructive pulmonary disease, and acute respiratory distress syndrome (Tao et al., 2022), but there are a few possible pathways linking them to AD development. Eosinophil cationic protein was shown to have amyloid-like aggregation capacity (Torrent et al., 2010). Furthermore, a persistent increase in eosinophils (such as that seen in hypereosinophilic syndrome) can induce neuropathic symptoms (Werner and Wolf, 1990; Brito-Babapulle, 2003) and even development of dementia. Fortunately, such symptoms resolve spontaneously after steroid therapy (case-study described by Kaplan et al., 1989). Eosinophil production is promoted by interleukin-3 (IL-3) (see the review by Tao et al., 2022 on the eosinophils pathophysiology), a cytokine that is released by astrocytes in the brain (just as *APOE*). IL-3 was previously shown to be heightened in AD patients carrying *APOE* risk genes (Soares et al., 2012). Additionally, IL-13 (released by eosinophils) belongs to a set of markers shown to correlate well with amyloid presence indicated on PET scans (Kiddle et al., 2012). Also, eosinophil recruiting chemokine (CCL-11/Eotaxin-1, produced by microglia among other cells) was linked to cognitive and executive function impairments, and was even named "Endogenous Cognition Deteriorating Chemokine" or "Accelerated Brain-Aging Chemokine" (Ivanovska et al., 2020). In the studies testing AD patients (i.e., people with clinical signs of dementia, not necessarily with defined risk-genes burden) granulocyte counts give variable results. Patients seem to have lower (Shad et al., 2013; Chen et al., 2017) or higher (Lunnon et al., 2012) levels of basophils, slightly lower levels of eosinophils (Järemo et al., 2013; Chen

et al., 2017), and elevated (Shad et al., 2013; Huang et al., 2022) or similar (Chen et al., 2017) neutrophils compared to the healthy controls. In our and Zhang studies, granulocytes were elevated in healthy middle age or older participants with detected AD risk but no symptoms of dementia. Thus, the role of granulocytes in the very early development/initiation of AD is possible, but their involvement in the course of the disease appears to be complex. Perhaps selected granulocytes are elevated in response to systemic or low-grade inflammation, reflecting pro-inflammatory features of the *APOE*-ε4 variant (Tzioras et al., 2019), which impacts on CSF function and promotes disease (Stock et al., 2018). *APOE*-ε4 plays a distinct role in many aspects of AD-related inflammation, including Aβ clearance, changes in microglia and glial cell functions, brain metabolism, autophagy, disruption of intracellular inflammatory pathways, and BBB permeability (Kloske and Wilcock, 2020). Why would the number of eosinophils be reduced in AD patients? Eosinophils are recruited from the blood to the infected/inflamed tissue (Miyabe et al., 2021). Maybe during the later stages of AD this process is intensified and tissue/brain eosinophilia is not reflected in the blood of AD patients.

The influence of risk genes on LOAD incidence is substantial, but not decisive. In the case of our study, we do not know which of our participants (now middle-age, healthy individuals) will develop dementia in the future. There are several blood parameters that have been identified as potential risk factors for AD and while these factors do not directly cause AD, we believe that they may contribute to the development or progression of the disease. However, we do not know if the blood cell differences observed in this study will persist in aging and will correlate with the AD onset and development. We found no other differences in blood tests in our healthy population that are sometimes reported in AD patients (within hematocrit, hemoglobin, platelet counts etc. (Chen et al., 2017). The resource-modulation hypothesis states that deteriorating effects of genetic variants (Lindenberger et al., 2008) are balanced by available reserve resources early in life, and some effects may be undetectable until people age and lose the reserves. The impact of risk genes on various functions and health can thus vary over a lifetime. A sample size of A+P+ and A+P- groups is a certain limitation in our study and replication (at best in multiple populations) would validate obtained results. However, an undoubted strength of our study is the control and balancing of additional risk factors (demographic, health), although some of the factors were collected through questionnaires and not measured objectively.

CONCLUSIONS

Healthy middle-aged carriers of *APOE* and *PICALM* AD risk alleles were characterized by slightly modified granulocyte profile: slightly elevated eosinophil levels and subtle group-dependent alternations in basophils levels as compared to non-risk carriers. Longitudinal observation is needed (and is planned) to find out which of the participants will eventually show signs of dementia. When validated, the blood cell profiles, along with other tests, could be included in multi-factor tests for early dementia risk assessment. It could be beneficial in regard to personalized health care, as there are many AD risk factors that may contribute differently depending on the individual circumstances of each person. Studying the impact of risk genes on healthy individuals can potentially lead to the discovery of diagnostic biomarkers that can identify individuals who are at risk of developing AD before the onset of symptoms. This could be critical for early intervention and treatment, which may help slow down or even prevent the progression of the disease.

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SUPPLEMENTARY MATERIALS

Table S1. Granulocytes laboratory norms.

Granulocytes type	Laboratory norm [K/µl]
Eosinophils	0.02-0.50
Neutrophils	2.00-7.00
Basophils	0.02-0.10

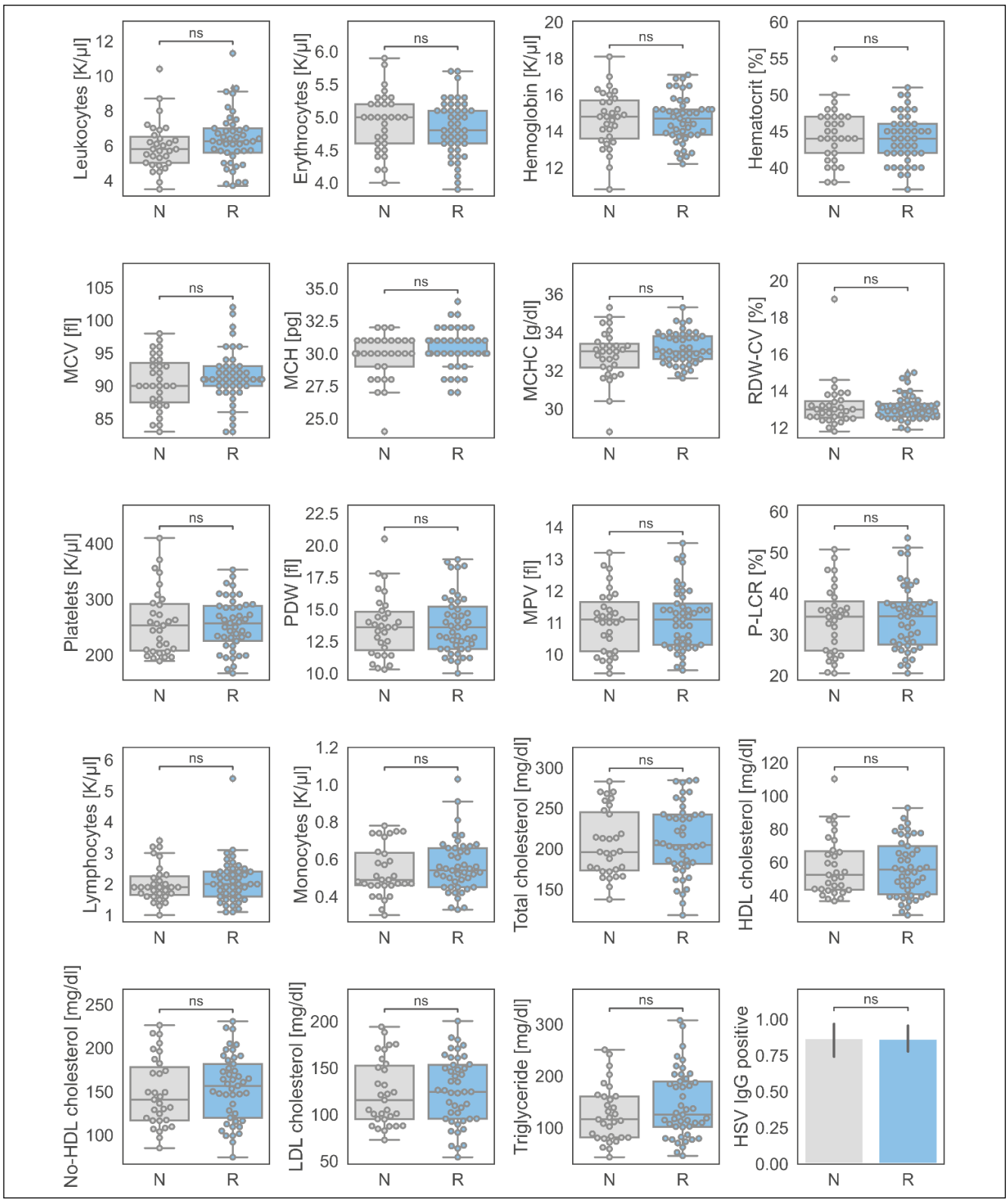

Fig. S1. Summary of exploratory analysis of other scores from blood count and lipoprotein panels – main effect of APOE genotype. Risk carriers (R) *vs.* non-risk group (N). The graph shows box plots with an additional representation of the distribution of individual data points denoted by grey dots. The central horizontal bar shows the median, and lower and upper boundaries show the 25th and 75th percentiles. MCV – mean corpuscular volume, MCH – mean corpuscular hemoglobin, MCHC – mean corpuscular hemoglobin concentration, RDW-CV – red blood cell distribution width, PDW – platelet distribution width , MPV – mean platelet volume, P-LCR – platelet-large cell ratio, HSV – herpes simplex virus. | ns – not significant.

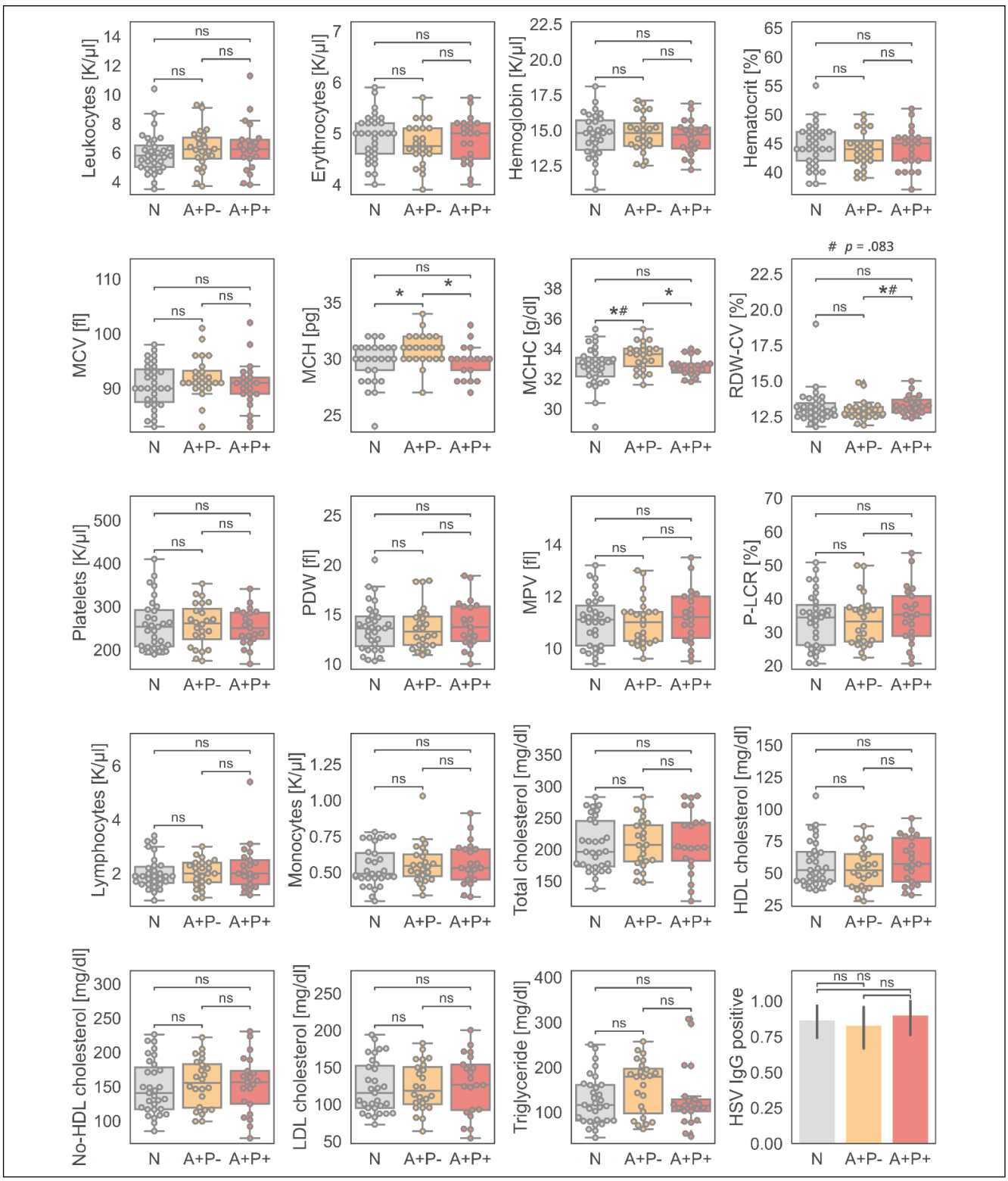

Fig. S2. Summary of exploratory analysis of other scores from blood count and lipoprotein panels – comparisons after dividing the R group to single-*APOE* risk (A+P-) and *APOE&PICALM* double-risk (A+P+). The graph shows box plots with an additional representation of the distribution of individual data points denoted by grey dots. The central horizontal bar shows the median, and lower and upper boundaries show the 25th and 75th percentiles. MCV – mean corpuscular volume, MCH – mean corpuscular hemoglobin, MCHC – mean corpuscular hemoglobin concentration, RDW-CV – red blood cell distribution width, PDW – platelet distribution width , MPV – mean platelet volume, P-LCR – platelet-large cell ratio, HSV – herpes simplex virus. | * p<0.05, *# significant result before the Holm-Bonferroni correction; ns – not significant. # Trend levels indicated by exact *p*-values.