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SIGNIFICANCE OF THE RELATIONSHIP BETWEEN PROINFLAMMATORY CYTOKINE GENE POLYMORPHISMS AND PITUITARY FOLLICULOSTELLATE CELLS FOR HUMAN LONGEVITY

ZNAČAJ ODNOSA IZMEĐU POLIMORFIZAMA GENA PROINFLAMATORNIH CITOKINA I FOLIKULOSTELATNIH ĆELIJA HIPOZIFE ZA DUGOVEČNOST ČOVEKA

Jovana Čukuranović Kokoris[1], Tatjana Jevtović Stoimenov[1], Dragan Bogdanović[2], Rade Čukuranović[1], Jasen Kundalić[1], Žaklina Šmelcerović[1], Miljana Pavlović[1], Neda Milenković[3]

[1]University of Niš, Faculty of Medicine, Niš, Serbia
[2]State University of Novi Pazar, Novi Pazar, Serbia
[3]University of Belgrade, Pharmaceutical Faculty, Belgrade, Serbia

Summary

Background: The focus of this study was on the analysis of the relationships between polymorphisms of proinflammatory cytokine genes (tumour necrosis factor α-TNF-α; interleukin 6-IL-6; and interleukin1β-IL-1β), morphology of non-endocrine pituitary folliculostellate cells (FS), gender, and age.
Methods: The research involved 20 cadavers (10 male and 10 female) divided into two groups: from 51 to 70 years of age (group I) and over 70 years of age (group II). Pituitary glands were collected during routine autopsy. Isolated pituitaries of cadavers of both sexes were prepared using routine histological techniques. On 5-μm-thick pituitary sections, FS cells were immunohistochemically stained using a primary polyclonal anti-S100 (1:400) antibody, visualised by DAB, and analysed by light microscopy at 40x magnification. Stereological analysis was performed using ImageJ. The PCR-RFLP method was used for genotyping single-nucleotide polymorphisms (SNPs) in TNFα, IL-1β, and IL-6.
Results: The morphology of FS cells in the second age group showed that they were irregularly shaped, more numerous, single or in groups and significantly ($p<0.05$) larger (2.23 times) compared to group I. Single-nucleotide polymorphism (SNP) analysis showed no correlation between SNPs of the examined genes and FS cell morphology.
Conclusion: In humans of both genders, the volume density of FS cells increases with age and is not associated with gene polymorphisms in TNFα, IL-1β, or IL-6.

Keywords: ageing, cadavers, folliculostellate cells, TNF-α, IL-1β, IL-6

Kratak sadržaj

Uvod: Fokus ove studije bio je na analizi odnosa između polimorfizama gena proinflamatornih citokina (faktor tumorske nekroze α-TNF-α; interleukin 6-IL-6; i interleukin 1β-IL-1β), morfologije neendokrinih folikulo-stelatnih (FS) ćelija hipofize, pola i starosti.
Metode: Istraživanje je obuhvatilo 20 kadavera (10 muškaraca i 10 žena) podeljenih u dve grupe: od 51 do 70 godina starosti (grupa I) i preko 70 godina starosti (grupa II). Hipofize su prikupljene tokom rutinske autopsije. Izolovane hipofize kadavera oba pola su pripremljene korišć enjem rutinskih histoloških tehnika. Na presecima hipofize debljine 5 μm, FS ćelije su imunohistohemijski obojene korišćenjem primarnog poliklonskog anti-S100 (1:400) antitela, vizualizovane DAB-om i analizirane svetlosnom mikroskopijom pri uvećanju od 40 puta. Stereološka analiza je izvršena korišćenjem programa ImageJ. Za genotipizaciju jednonukleotidnih polimorfizama (SNP) TNF-α, IL-1β i IL-6 korišćena je PCR-RFLP metoda.
Rezultati: Morfologija FS ćelija u drugoj starosnoj grupi pokazala je da su nepravilnog oblika, brojnije, pojedinačne ili u grupama i značajno ($p<0,05$) veće (2,23 puta) u poređenju sa grupom I. Analiza jednonukleotidnih polimorfizama (SNP) nije pokazala korelaciju između SNP-ova ispitivanih gena i morfologije FS ćelija.
Zaključak: Zaključeno je da se kod ljudi oba pola zapreminska gustina FS ćelija povećava sa godinama i nije povezana sa genskim polimorfizmima TNFα, IL-1β i IL-6.

Ključne reči: starenje, kadaveri, folikulostelatne ćelije, TNF-α, IL-1β, IL-6

Address for correspondence:

Jovana Čukuranović Kokoris, PhD
Department of Anatomy, Faculty of Medicine, University of Niš
81 Dr Zorana Đinđića Boulevard, 18000 Niš, Serbia
Phone: +381 64 529 55 76
e-mail: jovana.c85@gmail.com;
jovana.cukuranovic.kokoris@medfak.ni.ac.rs

Introduction

The biological process of ageing is inflammatory in nature, during which there is increased secretion of cortisol (1), as well as inflammatory components (2) in the blood. Elevated cortisol levels during ageing result in slowed T-cell proliferation, telomere shortening, thymus atrophy (3), increased C-reactive protein (CRP) and pro-inflammatory cytokines (4), and decreased levels of anti-inflammatory cytokines (5).

Although folliculostellate (FS) cells, anterior pituitary cells, are non-endocrine, agranular, and stellate-shaped, which do not produce any pituitary hormone, they are considered to be functional support structures for endocrine cells (6). Research by Le Tissier and Mollard (7) demonstrated that anterior pituitary cells communicate with one another. Also, an important role in communication is played by FS cells, named »excitable cells«, because they have numerous signalling mechanisms for generating and transmitting messages over long distances. Earlier studies on isolated endocrine cells showed that they generate action potentials that increase cytosolic calcium concentration. It is supposed to be a way of adaptation of numerous cellular functions, such as gene expression and exocytosis (8).

Cytokine levels are affected by single-nucleotide polymorphisms (SNPs) in genes encoding IL-1β, IL-6, and TNF-α, which can significantly influence the process of pathogenic ageing (9). Polymorphisms in the TNF-α promoter region at position-308 (rs1800629) regulate TNF-α production (10). Higher TNF-α production is associated with the A allele, which may lead to immunological disorders and increased mortality in autoimmune and infectious diseases (11). Microglia and astrocytes in the brain produce proinflammatory interleukin-1β (IL-1β) (12). IL-1β gene expression can be affected by the 31T>C promoter polymorphism rs16944 (13), in which a substitution at position -31 T in Cat disrupts the TATA box, changing TATAAA to CATAAA (14). This polymorphism has been associated with an increased risk of developing certain inflammatory diseases (15). IL-6 is a cytokine that is released in response to trauma, infection, neoplasia, and burns, and is expressed by a wide range of cells (16). Increased IL-6 protein expression was observed in the G/G genotype of the IL-6 rs1800795 (G-174C) gene polymorphism compared to the C/C genotype (17). The -174 G/C variant is associated with chronic obstructive pulmonary disease (18), cardiovascular disease (19), microvascular complications of diabetes mellitus (20), obesity comorbidities (21), and also with longevity (22).

Taken together, based on the literature on FS cells and inflammation that occurs in the aging process, we wanted to determine the relationship between immunohistomorphometric characteristics of FS cells in relation to polymorphisms of genes encoding proinflammatory cytokines: IL-1 T(-31) C (IL-1 production: T<C), IL-6 G(-174)C (IL-6 production: C<G) and TNF-G (-308)A(TNF-α production: G<A).

Materials and Methods

The decision of the Ethics Committee of the Faculty of Medicine, University of Niš (No. 12-2307-2/8 of March 10, 2016) allowed the collection of pituitary glands from cadavers of both sexes, which were processed in this study. The pituitary glands were collected during routine autopsy, at the Centre for Forensic Medicine in Niš, Serbia, up to 24 hours after death. It was established that the cadaveric subjects used in our research had no known diagnoses of neurological or endocrine diseases, psychiatric disorders, nor had any brain or pituitary damage during life. The collected pituitary glands were weighed and fixed in 10% formalin for further histological processing (23). The study involved 20 cadavers (10 men and 10 women) divided into two groups according to their age: the first group (I) included cases from 51 to 70 years old, and the second group (II) included cases of 71 years old and older.

Immunohistochemistry and stereological analysis

The extracted pituitary glands of women's and men's cadavers were embedded using an established histological technique (24) and immunohistochemically labelled with the primary polyclonal antibody S-100 (DAKO, Denmark), as previously described in detail (25). Immunoreactive FS cells were imaged with a 1.3 megapixel digital camera on a light microscope (Carl Zeiss GmbH, Vienna, Austria) at 40x magnification, then morphometrically analysed. Counting was performed with the M_{168} multipurpose test system on 30 fields of view from the dorsal and ventral sides, i.e., a total of 60 fields of view per analysed case, using the ImageJ software (http://imagej.nih.gov/ij/) (24, 25). Calculation of the V_V of immunopositive FS cells has been described in detail previously (24).

Blood samples and DNA isolation

Blood samples from cadaver iliac vessels were used to isolate genomic DNA using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany), as recommended by the manufacturer. The obtained DNA samples were stored at -20 °C until genotyping analysis was performed.

Genotyping

The rs1800629 TNF-α -308 G/A SNP, rs1800795 IL-6 -174G/C and rs1143627 IL-1β -31 T>C were determined using the polymerase chain reaction – restriction fragment length polymorphism (PCR–RFLP) technique. PCR was performed in a 25 μL volume reaction containing 20 ng of DNA, 12.5 μL KAPA2G Fast HotStart ReadyMix (Kapa Biosystems Inc, CITY, USA) and 20 pmol of each primer: TNF-α -308 forward primer: 5′-AGG CAA TAG GTT TTG AGG GCC AT-3′, reverse primer: 5′-ACA CTC CCC ATC CTC CCT GCT-3′; IL-6 -174 forward primer: 5′-TTGTCAAGACATGCCAAGTGCT-3′; reverse primer: 5′-GCCT CAG AGA CAT CTC CAG TCC-3′; IL-1β forward primer 5′-AGAAGCTTCCACCAATACT -3′; reverse primer 5′-TAGCACCTAGTTGTAAGGA-3′.

For the determination of SNPs rs1800629 TNF-α -308 G/A, rs1800795 IL-6 -174G/C and rs1143627 IL-1β -31 T>C, the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) technique was used in volume of 25 μL (20 ng DNA, 12.5 μL KAPA2G Fast HotStart ReadyMix (Kapa Biosystems Inc, CITY, USA)) and 20 pmol of each primer: TNF-α -308 forward primer: 5′-AGG CAA TAG GTT TTG AGG GCC AT-3′, reverse primer: 5′-ACA CTC CCC ATC CTC CCT GCT-3′; IL-6-174 forward primer: 5′-TTGTCAAGACATGCCAAGTGCT-3′; reverse primer: 5′-GCCT CAG AGA CAT CTC CAG TCC-3′; IL-1β forward primer 5′-AGAAGCTTCCACCAATACT -3′; reverse primer 5′-TAGCACCTAGTTGTAAGGA-3′ (26).

The PCR was performed as follows: initial denaturation occurred at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 15 s, ending with a final extension at 72 °C for 1 min (27). Restriction digestion using NcoI, NlaIII, and Alu I restriction enzymes at 37 °C overnight (Fermentas GmbH, St. Leon-Rot, Germany) was performed after amplification of the DNA segments of interest and verified by 2–4% agarose gel electrophoresis. Restriction endonuclease for the TNF α gene generated two fragments, 97bp and 20 bp, in the wild type (GG) and one, 117 bp, fragment in the polymorphic (AA) genotype. Overnight digestion of IL6, PCR products with 3 units NlaIII at 37 °C yielded for the G allele 3 fragments of 244, 133, and 11 bp in length, and for the C allele also 3 fragments of 133, 111, and 56 bp in length. The obtained restriction fragments were analysed by 8% polyacrylamide gel electrophoresis (PAGE) and visualised under UV light. Two independent researchers accomplished separate genotype analyses (28).

Statistical analysis

In all analyses, the cut-off for statistical significance was set at the 0.05 (5%) assessment error. Comparisons of mean values of volume density and age between males and females and between the two age groups of examinees were done using the Student's t-test. Linear regression analysis was used to examine the relationship between age and volume density. Genotype and allele frequencies in the studied cases were tested using the chi-square (χ^2) test. Obtained frequency values were compared to the values predicted by the Hardy–Weinberg equilibrium (HWE) using the χ^2 test. Genetic risks were additionally estimated by odds ratios (OR) with 95% confidence intervals (95% CI). SPSS version 16.0 statistical software package (SPSS Inc, Chicago, Illinois, USA) was used for analysis (25).

Results

Morphological analysis

In the first group of men and women cadavers, S-100 immunopositive FS cells were star-shaped, extremely rare, single, and scattered throughout the anterior pituitary (*Figure 1A, B*). Long, thin extensions of these cells extended between endocrine cells and were interconnected (*Figure 1B*). In older cadavers of group II, in both sexes, the shape of FS-immunopositive cells did not change, but their number was higher than in the first group (*Figure 1C, D*). In this group, in addition to individual cells, there are also FS cells clustered (*Figure 1C, D*). Numerous follicles can be observed in Group II cadavers with FS cells located around some of them (*Figure 1E*), while some of them are negative for S-100 (*Figure 1F*). *Table I* shows the results of the stereological analysis for all evaluated cases.

The mean age of the male (66.60±11.26) and female (72.40±10.00) cases did not differ significantly ($t=1.218$, $p=0.239$). In all 20 analysed cases, the mean volume density of FS cells was 8.30±4.80%. In 10 male cadavers, the average FS cell volume density (7.75±5.14%) was lower ($p>0.05$) than in 10 female cadavers (8.85±4.65%). Average V_{VFS} cell in group II was higher ($p<0.05$) by 122.9% compared to group I (*Table II; Figure 2A*). Further, linear regression analysis performed in 20 cases demonstrated a statistically significant association between age and volume density (*Figure 2B*). The association was established using the regression model: $V=0.210 \times age - 6.314$, which explained 18% of the variance in volume density (Adjusted $R^2=0.180$), indicating a moderate effect of age on volume density.

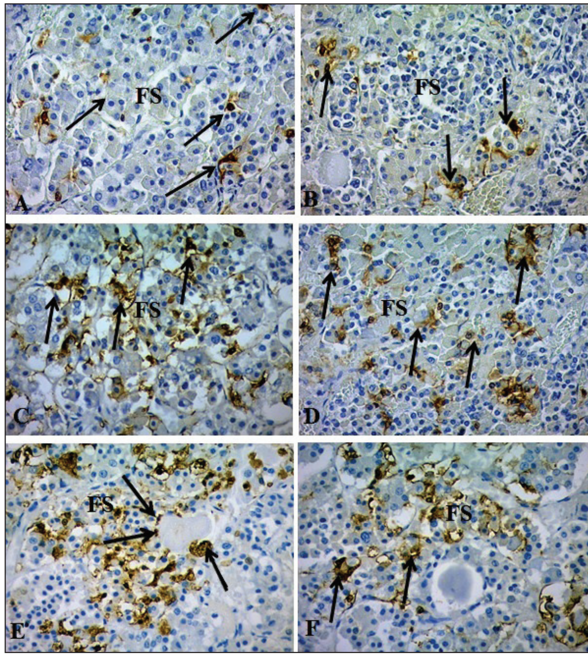

Figure 1 Representative photomicrographs of individual immunopositive S-100 FS pituitary cells of a men (A) and women (B) cadavers aged 53 years; numerous single or clustered S-100 positive FS cells from 73-year- old men cadaver (C) and 71-year-old women cadaver (D); S-100 immunoreactive FS cells (arrows) within the anterior pituitary follicle in a 73-year-old men case (E); follicle in a 71-year-old women cadaver not coated with S-100 antibody (F). PAP method, objective magnification 40x.

Table I The results of morphometric measurements for all corpses.

Corpses	Years	♂ ♀	Groups	V_{VFS} (%)
1	51	Male	I	3.43
2	54	Male	I	6.50
3	54	Female	I	6.23
4	56	Male	I	4.77
5	61	Male	I	4.93
6	61	Female	I	3.90
7	64	Male	I	3.67
8	66	Female	I	2.63
9	67	Male	I	2.97
10	69	Female	I	12.33
11	71	Female	II	18.77
12	73	Male	II	19.17
13	76	Female	II	11.70
14	78	Male	II	10.40
15	79	Female	II	9.50
16	80	Male	II	9.33
17	81	Female	II	8.93
18	82	Male	II	12.33
19	83	Female	II	6.87
20	84	Female	II	7.60

Table II Average age and volume density of folliculostellate cells in the studied groups.

Characteristic	Parameter	I group	II group	Total
Years	Mean	60.30	78.36	69.76
	SD	6.25	4.20	10.58
V_{VFS} (%)	Mean	5.14	11.46	8.30
	SD	2.84	4.29	4.80

V_{VFS} (%) – volume density of folliculostellate cells. Mean – mean value of the volume density of FS cells; SD – standard deviation

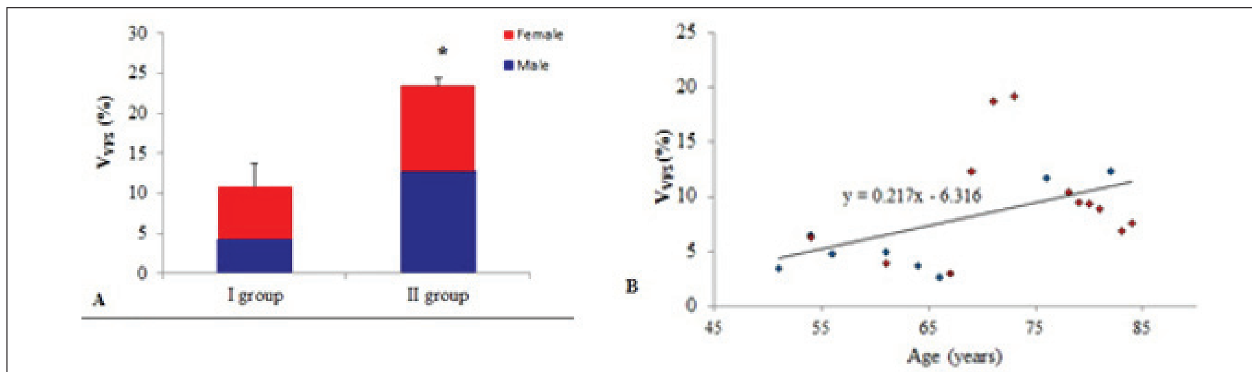

Figure 2 The graphical representation of the relative volume density (V_{VFS}, %) measured in FS cells in cadaver cases from 51 to 70 years old (I group), and cases of 71 years and older (II group) (A); association between age and volume density of FS cells in 20 studied cases (B). All values are the means ± SD. *$p<0.05$ I group vs II group. V_{VFS} (%) – volume density of FS cells.

Genotyping analysis

Due to the low frequency of polymorphic genotypes, GA and AA, GC and CC and TC and CC genotypes were grouped in each case group. Group I included 10 healthy adults (6 men and 4 women) for the TNF-α gene; 6 were wild-type homozygous (GG genotype) carriers (60%), and 4 were carriers of genotypes containing variant A allele (GA or AA genotype) (40%). For the IL-6 gene 6 were wild-type homozygous (GG genotype) carriers (60%) and 4 were carriers of genotypes containing variant C allele (GC genotype) and for IL-1β gene 7 were wild-type homozygous genotype (TT genotype) carriers (70%) and 3 were carriers of heterozygous genotype (TC genotype). Group II included 10 adults (4 men and 6 women); for the TNF-α gene, 6 were wild-type homozygous (GG genotype) carriers (60%), and 4 were carriers of heterozygotes (GA genotype) (40%). For the IL-6 gene, 7 were wild-type homozygous (GG genotype) carriers (70%), and 3 were carriers of genotypes containing variant C allele (GC genotype) (30%), and for IL-1β gene, 8 were wild-type homozygous (TT genotype) carriers (80%), and 2 were carriers of heterozygotes (TC genotype) (20%). Distribution of the rs1800629 TNF-α -308 G/A SNP, rs1800795 IL-6 -174 G/C and rs1143627 IL-1β T>C genotypes in both groups of studied cases is shown in *Table III*.

Table IV shows the distribution of alleles rs1800629 TNF-α -308 G/A SNP, rs1800795 IL-6 -174 G/C and rs1143627 IL-1β T>C in all groups of cases.

Association of the average value of V_V (%) FS cells and distribution of the rs1800629 TNF-α -308 G/A SNP, rs1800795 IL-6 -174 G/C, and rs1143627 IL-1β T>C genotypes in all studied cases are presented in *Table V.*

The average value of volume density is higher in 13 cases with a homogeneous genotype (GG) than in 7 cases with a heterogeneous genotype (GC) (8.69±4.65: 7.57±5.36%, respectively), but the t-test did not confirm that the difference between these values is statistically significant (t=0.487 and p=0.632). The average value of volume density is lower in 12 cases with a homogeneous genotype (GG) than in 8 cases with a heterogeneous genotype (GA) (7.60±4.71: 9.33±5.07%). Still, the t-test did not confirm that the difference between these values is statistically significant (t=0.776, p=0.448). The average value of volume density is higher in 14 cases with homogeneous structure (TT) than

Table III Distribution of TNF-α -308 G/A, IL-6 174 G/C and IL-1β -31 T/C alleles by groups.

	Genotype TNF-α		Genotype IL-6		Genotype IL-1β	
	GG	GA+AA	GG	GC+CC	TT	TC+CC
Group I (51-70 years old)	6	3 + 1	6	4 + 0	7	3 + 0
Group II (>71 years old)	6	4 + 0	7	3 + 0	8	2 + 0

Table IV Distribution of TNF-α -308 G/A, IL-6 174 G/C and IL-1β -31 T/C alleles by groups.

	Allele TNF-α		Allele IL-6		Allele IL-1β	
	G	A	G	C	T	C
Group I (51–70 years old)	15	5	16	4	17	3
Group II (>71 years old)	16	4	17	3	18	2

Table V Association of the mean value of volume density of folliculostellate cells (V_{VFS} (%)) and distribution of TNF-α, IL-6, and IL-1β genotypes in all study cases.

	Genotype TNF-α		Genotype IL-6		Genotype IL-1β	
	GG 12	GA+AA 8	GG 13	GC+CC 7	TT 14	TC+CC 6
V_{VFS} (%)	7.6±4.7	9.3±5.1	8.7±4.6	7.6±5.4	8.5±5.1	7.8±4.2
	t=0.776 p=0.448		t=0.487 p=0.632		t=0.243 p=0.810	

Table VI Correlation between volume density of folliculostellate cells (V_{VFS} (%)), age, sex, IL-1β, IL-6 and TNF-α genotypes.

Characteristic	Parameter	Age	Female sex	IL-1β	IL-6	TNF-α
V_{VFS} (%)	r	0.421	0.260	0.038	-0.173	0.204
	p	0.023	0.173	0.874	0.466	0.389
Age	r		0.120	-0.170	-0.091	-0.124
	p		0.536	0.473	0.703	0.602
Female sex	r			-0.218	0.105	0.204
	p			0.355	0.660	0.388
IL-1β	r				-0.023	-0.356
	p				0.924	0.123
IL-6	r					-0.171
	p					0.471

* ρ-Spearman's correlation coefficient.

in 6 cases with heterogeneous structure (TC) (8.47±5.16:7.89±4.25%). Still, the t-test did not confirm that the difference between these values is statistically significant ($t=0.243$, $p=0.810$).

Multivariate regression analysis with the use of the enter method (unconditionally keeps all entered independent variables in the model, regardless of whether they are significantly related to the dependent variable or not) singles out age as the single significant predictor of volume density (B=0.217; 95% CI: 0.006 to 0.429; $p=0.045$). Multivariate regression analysis using the stepwise method (all possible combinations of independent variables are tested until the best solution is obtained) excludes genotyping from the model and identifies age as the only significant predictor of volume density (B=0.210; 95% CI: 0.016 to 0.405; $p=0.036$).

The correlation between age, sex, FS cell volume density, TNF-α, IL-1β, and IL-6 genotypes is shown in *Table VI*. A significant unfavourable correlation was observed between age and FS cell volume density ($\rho=0.421$, $p=0.023$), while there were no significant associations between age and sex, including TNF-α, IL-1β, and IL-6 genotypes ($p>0.05$).

Discussion

Cardiovascular diseases, metabolic syndrome, atherosclerosis, osteoporosis, type 2 diabetes, sarcopenia, and decline in cognitive abilities are companions of ageing (29). In earlier works, it was noted that ageing is accompanied by numerous inflammatory processes during which cytokines are activated: IL-1, IL-6, TNF-α, IL-1Ra, IL-12, IFFranceschN-a, CRP, IFN-b, sTNFR, and serum amyloid A, characteristic for inflammatory processes (30), which activate the neuroendocrine response of the HPA axis (31). In this study, it was shown that the stereological parameters of FS cells significantly increased with ageing in both sexes and that they did not change their stellate shape, in agreement with previously published work (24, 32). Another study showed a similar increase in the number of FS cells in people of both sexes after age 80. Pavlović et al. (6) noted an increase in the number of these cells in the mucoid wedge of the human pituitary gland, which was accompanied by an increased level of IL- 6 (7). It is assumed that FS cells (33). However, they are not endocrine; they exert a paracrine effect on other pituitary cells via their stellate extensions, thereby modulating their response to hyperactivity driven by the inflammatory process in older adults of both sexes (31).

In the normal brain, evidence strongly suggests that the »proinflammatory« cytokines IL-1β and TNF-α were constitutively expressed and are modulators of neuronal activities (34). Genetic variations in the genes encoding the investigated cytokines may be responsible for the appropriate cytokine profile and, therefore, could be important in the genetic regulation of ageing. Our study demonstrated no significant difference in the distribution of rs1800629 TNF-α -308 G/A SNP, rs1800795 IL-6 -174G/C, and rs1143627 IL-1 (T>C) alleles between group I and II of the studied cases. Serum TNF-α levels have been reported to increase with age in older adults with atherosclerosis. They are associated with mortality (35). The A allele of the TNF-α gene 308 G/A polymorphism, according to genetic studies, is associated with an increased risk of myocardial infarction. In contrast,

an increased risk of Alzheimer's disease is associated with TNF-α polymorphisms (36).

The problems associated with ageing are a constant focus of researchers seeking ways to mitigate the changes it causes. In the development of many diseases associated with ageing, the proinflammatory cytokine IL-6 plays a role, which is why it has been called the »gerontological cytokine« (37). A study by Bonafé et al. (38) on centenarian men and women from Italy showed that the proportion of homozygotes for the G allele at the -174 locus is decreasing in males, but not in females. The same study found that among males, only homozygotes for the G allele at the -174 locus showed higher serum IL-6 levels than C allele carriers, and that males homozygotes for the -174 locus GG were at a disadvantage in terms of longevity (38). Previous studies have shown that dysregulation of proinflammatory cytokine (IL-6) is the first step in the development of chronic diseases (39), as demonstrated in patients with cirrhotic portal hypertension (40). Sorensen et al. (41), based on a meta-analysis of longevity in a large cohort of Danish and Dutch populations older than 85 years, found that longevity was associated with carriers of the lower IL-6 cytokine-producing allele.

Macrophages during defence reactions produce proinflammatory cytokines of the interleukin-1 family, which have pronounced inflammatory and immunoenhancing effects. It is assumed that the physiological role of IL-1 is associated with the development of adaptive immunity in vertebrates, and that it acts by binding to specific receptors on the plasma membrane of target cells (42). Polymorphisms in the IL-1 gene have been shown to shorten life expectancy in older men in Sweden (43). Diseases characteristic of ageing may be associated with variants of the IL-1 gene, and regulation of inflammation may be alleviated by recombinant drugs, such as IL-1Rα blockers (44). However, in other studies, results are contradictory, and SNP frequencies vary strikingly across geographic and ethnic groups.

Conclusions

The results of our research, based on which we draw our conclusion, indicate that the volume density of pituitary FS cells of both sexes in humans increases with age and that the SNPs studied genes for IL-1β (31C/T), IL-6 (-174C/G) and TNF-α (308A/G) are not associated with observed changes in FS cells with ageing.

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Limitations and future directions

In this study, we aimed to assess changes in FS cells with age and their associations with targeted genetic polymorphisms in genes coding cytokines. The main limitation of our study was the small number of cases (20 in all), subdivided into 2 groups based on age. Also, 1 SNP per gene was analysed, and other SNPs in the candidate genes may be associated with FS morphological changes. Future studies should include a much higher number of cases and known SNPs in the candidate gene should be analysed.

Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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