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**Morphometric analysis of somatotropic and folliculostellate cells of
human anterior pituitary during ageing**

Морфометријска анализа соматотропних и фоликулостелатних ћелија
аденохипофизе човека током старења

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Morphometric analysis of somatotropic and folliculostellate cells of human anterior pituitary during ageing

Морфометријска анализа соматотропних и фоликулостелатних ћелија аденохипофизе човека током старења

SUMMARY

Introduction/Objective In this study, we have pointed out the immunohistomorphometric characteristics of somatotropic (GH) and folliculo-stellate (FS) cells of the human pituitary gland during ageing.

Methods On histological sections of the pituitary gland of 14 male cadavers of different ages, the GH and FS cells were immunohistochemically labeled with corresponding antibodies, monoclonal anti-GH antibody and polyclonal anti-S100 antibody, respectively. Immunopositive GH- and FS-cells were further morphometrically analyzed using *ImageJ* software.

Results The obtained results of morphometric analysis showed that the surface area of GH cells increased significantly with age. In these cells, the nuclear-cytoplasmic ratio gradually decreased and became significantly higher after the age of 70 years. The volume density of GH cells has not changed during ageing, while in FS cells this parameter significantly increased in the cases older than 70 years. The nuclear-cytoplasmic ratio of GH cells is negatively correlated with the volume density of FS cells.

Conclusions Based on the obtained results, we concluded that hypertrophy of GH and FS cells occurs in men with ageing and that correlation between the morphometric parameters of these two cell types indicates their mutual interaction.

Keywords: ageing; men; GH cells; folliculostellate cells; immunohistomorphometry

САЖЕТАК

Увод/Циљ У овој студији смо указали на имунохистоморфометријске карактеристике соматотропних (ГХ) и фоликулостелатних (ФС) ћелија хипофизе човека током старења.

Метод На хистолошким пресецима хипофизе 14 мушких кадавера различите старости, ГХ и ФС ћелије су имунохистохемијски обележене одговарајућим антителима (моноклонско анти-ГХ антитело и поликлонско анти-С100 антитело). Имунопозитивне ГХ- и ФС-ћелије су морфометријски анализирани коришћењем *ImageJ* софтвера.

Резултати Добијени резултати морфометријске анализе су показали да се површина коју заузимају ГХ ћелије значајно повећава са старењем. У овим ћелијама нуклеарно-цитоплазматски однос се постепено смањивао и након 70. године старости постао значајно већи. Волуменска густина ГХ ћелија се није мењала током старења, док је код ФС ћелија овај параметар значајно повећан у случајевима старијим од 70 година. Нуклеарно-цитоплазматски однос ГХ ћелија је у негативној корелацији са волуменском густином ФС ћелија.

Закључак На основу добијених резултата, закључили смо да се хипертрофија ГХ и ФС ћелија јавља код мушкараца са старењем и да корелација између морфометријских параметара ова два типа ћелија указује на њихову међусобну интеракцију.

Кључне речи: старење; мушкарци; ГХ ћелије; ФС ћелије; имунохистоморфометрија

INTRODUCTION

It is well known that ageing brings with it various physiological changes within the human organism, which are especially pronounced in the functioning of the somatotropic and reproductive axis. Decreased secretion of growth hormone (GH) with ageing is referred to as somatopause [1]. The basis of somatopause is multiple neuroregulatory collapse, such as the lack of secretion of growth hormone (regulated by growth hormone releasing hormone from the hypothalamus), ghrelin and insulin-like growth factor 1 (IGF-1), as well as excessive secretion of somatostatin [1, 2].

This ultimately results in a reduced amount of secreted GH per one secretory pulse. Ageing does not have an impact on the frequency of pulsatile secretion of GH, basal secretion of GH, half-life of GH and its elimination kinetics [3]. These changes in GH secretion patterns with ageing are probably in part the consequence of certain structural changes at the hypothalamic level [4], and in part the consequence of changes in anterior pituitary somatotrophic cells, which have not been sufficiently studied so far [5]. In contrast to hypogonadism, the negative feedback mediated by GH and/or IGF-1 becomes stronger with advancing age. The consequence of this phenomenon is the biochemical hyposomatotropism with an exponential decline of GH and IGF-1 concentrations starting from early adult age, which clinically manifests in the form of osteopenia, sarcopenia, intraabdominal obesity, insulin resistance, hyperlipidemia, increased risk for atherosclerosis and lower quality of life [1, 6].

The knowledge of the function of hypothalamo-somatotropic axis during ageing has led to therapeutic use of GH and other hormones in the elderly as certain 'elixirs of youth'. However, the expected results have not been obtained, and the incidence of adverse effects and potential malignancy risks in the elderly have led endocrinologists to decide that there are no valid reasons for clinical use of GH to reverse age-related changes [7]. Naturally, future research of the pathways responsible for GH deficiency during ageing should hopefully resolve the present dilemmas as to the use of GH in the attempts to reverse ageing and to prolong human life.

In their fundamental work, Schwartz et al. [8] reported that the function of anterior pituitary probably relies on the integration of multiple received input signals, including hypothalamic, peripheral and intrapituitary, which may have stimulatory or inhibitory effects on the hormone production. Interactions of somatotrophic (GH) cells with the other hormonal and non-hormonal pituitary cells in healthy elderly appear to be very important [9, 10, 11]. Pituitary folliculostellate cells (FS) can release various products into the intercellular space, and thus can influence the functioning of neighboring hormone-producing cells [12].

The internal hormonal milieu of older men changes with advancing age [13], and we may well suppose that such changes can stimulate FS cell interactions with somatotropes via paracrine loops. These interactions can consequentially alter the function of somatotropes, which would ultimately result in age-related changes of GH levels in the plasma.

In view of the fact that hyposomatotropism during ageing can occur as the consequence of structural changes at all levels of the somatotropic axis and that age-related histomorphometrical changes at the level of anterior pituitary are insufficiently studied, the aim of our study was to detect and quantify the changes in morphology and density of human anterior pituitary somatotropic cells, using immunohistochemical and morphometric analysis in cases of different ages. We also tried to discover similar changes in adenohypophyseal folliculostellate cells, as the structures which, via paracrine pathways, could have an impact on the function of anterior pituitary somatotropic cells. Additionally, we evaluated statistically the possible association of the above changes during human ageing.

METHODS

Pituitary tissue sampling

The study material consisted of pituitary tissue samples taken from 14 men corpses, aged 41 to 87 years, which were divided into three groups. The first (I) group consisted of cases aged 41-49 years; the second (II) group implied cases aged 50-69 years and the third (III) group included cases older than 70 years.

Tissue samples were taken at a routine autopsy at the Center for Forensic Medicine in Niš, Serbia, with the approval of the Ethics Commission of the University of Niš, Faculty of Medicine (Decision No. 12-2307-2 / 8 of March 10, 2016), which is described in detail in our previous work [14].

Pituitary tissue processing and immunohistochemistry

Isolated pituitaries of the male corpses were histologically prepared according to the procedure described in detail earlier, while the morphometric analysis of GH and FS cells was performed according to the previously described procedure [15, 16]. The stained histological sections were then immunohistomorphometrically analyzed using light microscopy under 4x and 40x magnifications.

Morphometric analysis

Morphometric analysis was performed on digital images obtained by a 1.3 megapixel digital camera. Thirty visual fields were selected from both dorsal and ventral halves, *i.e.* 60 visual fields in total per each analyzed case. We obtained 10 visual fields each from both lateral anterior pituitary wings and 10 from the middle portion of both dorsal and ventral halves from all analyzed cases (20 visual fields in total from each lateral anterior pituitary wing and 20 from the middle portion in each analyzed case). Image analysis was performed using the *ImageJ* software (<https://imagej.nih.gov/ij/>).

As for GH immunoreactive cells, our analysis involved measurements of their area (A_{GH}) and the area of their nuclei (A_{NGH}). The nuclear-cytoplasmic ratio (N/C_{GH}) was calculated as the quotient of nuclear area and cytoplasmic area, with the cytoplasmic area obtained as the difference of area of the above cells and area of their nuclei. We performed the measurement of 60 somatotropic cells and 60 FS cells in the dorsal and ventral anterior pituitary halves in all analyzed cases (in total, 120 cells per a case).

The analysis was performed using the multipurpose test system M168 ($d=17.88$ μm , $a=15.49$ μm^2 , $AT=2601.54$ μm^2 , $LT=1501.92$ μm), placed over the analyzed digital image of histological sections. Volume density of GH (V_{VGH}) and FS cells (V_{VFS}) was obtained as the quotient of the number of dots in the test system which hit immunopositive cells (PF) and the total number of dots in the system ($PT=168$) per each analyzed field of the dorsal and ventral pituitary halves [17].

The values of area, nuclear area, nuclear-cytoplasmic ratio and volume density of GH cells and volume density of FS cells per each analyzed case were obtained as average values for all measured visual fields.

Statistical analysis

The statistical analysis was performed using the SPSS statistical software package (version 16). Dynamics of the values of morphometric parameters for the studied age groups was analyzed using the One Way ANOVA and Tukey-Kramer *post hoc* test. Due to a small

size of the analyzed sample, the obtained statistically significant differences were additionally verified by the calculation of the corresponding effect sizes.

RESULTS

Qualitative histological analysis

In younger individuals (the first group; age between 41 and 49 years), pituitary somatotropic cells were rare and scattered within the *pars intermedia*, while their presence was markedly greater in anterior pituitary lateral wings. They were predominantly polygonal, with eccentric euchromatic nuclei (with prominent nucleoli in some of the cells). A positive immunohistochemical reaction was observed in the cytoplasm of somatotropes, while in their nuclei the reaction was immunonegative (Figure 1A). Somatotropic cells in older cases (the third group; above 70 years) demonstrated a slightly stronger immunopositive reaction in the *pars intermedia* of anterior pituitary. In contrast to the above, in these cases there was a significant decline of the number of somatotropes in both *pars intermedia* and anterior pituitary lateral wings. Somatotropes were either single or in groups, located near the capillaries. The cells were larger, with an eccentric, hyperchromatic, immunonegative nuclei and occasional transparent cytoplasmic vacuoles (Figure 1B).

Folliculostellate cells in the anterior pituitary of younger cases (age between 41 and 49 years) were irregular to star-shaped in appearance. Their immunopositive cellular body has thin projections extending between endocrine cells. These cells were rare and irregularly distributed within the lateral wings, as well as in the mucoid wedge of the anterior pituitary (Figure 1C). The bodies of FS cells and their projections were visualized between endocrine cells. In cases over 70 years of age (the third group), we encountered a significantly increased number of FS cells in the middle and in the lateral wings of the anterior pituitary (Figure 1D). Although the irregular shape of their bodies made it difficult to estimate the size of the cells, it could be concluded that in general their bodies became larger and that the immunopositivity was stronger in the more advanced years of life.

Morphometric analysis

The results of morphometric analysis of anterior pituitary immunoreactive GH and FS cells in the studied cases are shown in Table 1.

The correlation analysis of age and morphometric parameters of GH immunoreactive cells showed that their area significantly increased ($R=0.7$; $p=0.005$; $N=14$) (Figure 2A), and nuclear-cytoplasmic index significantly decreased with advancing age ($R=-0.69$; $p=0.006$; $N=14$) (Figure 2B). Volume density of FS cells significantly increased during ageing ($R=0.80$; $p=0.001$; $N=14$) (Figure 2C). Nuclear area of GH immunoreactive cells did not change significantly with ageing ($p > 0.05$), while volume density of these cells increased with ageing, but the increase was not significant ($p > 0.05$). The results of bivariate linear regression additionally demonstrated that age was a significant predictor of area and nuclear-cytoplasmic ratio of GH immunoreactive cells and volume density of FS cells as well (Table 2). The factor of age was able to explain 49% of area variance, 48% of nuclear-cytoplasmic ratio of GH immunoreactive cells and 61% of volume density variance of adenohypophyseal FS cells. In all three instances, age represented a large effect size and could be shown using the models presented in Table 2.

A detailed dynamics of age-related changes in average values of morphometric parameters of adenohypophyseal immunoreactive GH and FS cells in the studied age groups was evaluated using the One Way ANOVA test. The results showed that the mean values of area and nuclear-cytoplasmic ratio of GH immunoreactive cells differed significantly between the studied age groups (Table 3). The average area of anterior pituitary GH immunoreactive cells significantly increased during the process of ageing ($F(2,11) = 4.77$, $p = 0.03$) (Figure 2D). The *post hoc* Tukey-Kramer test showed that this parameter had an increasing tendency during ageing, with the value in age group III being significantly higher compared to group I ($p < 0.05$), although not compared to age group II ($p > 0.05$). The average value of this parameter in age group II was higher than that in group I, but the difference was not statistically significant ($p > 0.05$). The average nuclear-cytoplasmic ratio of anterior pituitary GH immunoreactive cells significantly declined with ageing ($F(2,11) = 6.38$, $p = 0.01$) (Table 3, Figure 2E). The *post hoc* Tukey-Kramer test showed that this parameter had a declining tendency during ageing, with the value in age group III being significantly lower than that in group I ($p < 0.05$), but not compared to age group II ($p > 0.05$). The average value of this parameter in age group II was

lower compared to group I, but the difference was not statistically significant ($p > 0.05$). The average volume density of anterior pituitary FS cells ($F(2,11) = 5.08$, $p = 0.03$) significantly increased during the process of ageing (Table 3, Figure 2F). The *post hoc* Tukey-Kramer test showed that volume density of FS cells showed an increasing tendency with advancing age, with the values in age group III being significantly higher than those in age group I ($p < 0.05$), but not when compared to age group II ($p > 0.05$). The average value of this parameter in age group II was higher compared to age group I, but the difference was not statistically significant ($p > 0.05$). The remaining two analyzed morphometric parameters did not differ significantly between the studied age groups ($p > 0.05$).

The analysis of correlation between the morphometric parameters of GH immunoreactive and FS cells indicated the presence of a statistically significant negative correlation of average volume density of anterior pituitary FS cells and nuclear-cytoplasmic ratio of GH immunoreactive cells ($R = -0.71$, $p = 0.005$, $N = 14$). The linear regression analysis showed that volume density of FS cells in examined cases represented a statistically significant predictor of nuclear-cytoplasmic ratio of GH immunoreactive cells ($F(1,12) = 12.08$, $p = 0.005$), which could be represented using the following model: $(N/C)_{GH} = 0.287 - V_{VFS} \times 0.031$. In particular, the increased volume density of anterior pituitary FS cells was accompanied by a statistically significant decline of nuclear-cytoplasmic ratio of GH immunoreactive cells (Figure 2G), with the FS cell volume density of the studied cases being able to explain 46% of overall variance of nuclear-cytoplasmic ratio of GH immunoreactive cells ($R^2 = 0.46$), which represented a large size effect.

DISCUSSION

Ageing of the pituitary gland functionally manifests by its declining secretory activity, especially affecting the levels of growth hormone, prolactin (PRL) and thyroid-stimulating hormone (TSH) in the blood. These changes lead to so called age-related diseases, which predominantly affect the target organs of these hormones [6, 18]. Some previous studies detailly reported a fall in different parameters of somatotrophic cells with ageing [1]. In the study conducted by Sano et al. [19], the presence of interstitial, perivascular fibrosis was documented semiquantitatively: the fibrosis progressed with time and involved anterior pituitary

parenchyma in 88% of older individuals (predominantly males). The study reported a declining number of somatotropes in the lateral portions of the organ with ageing, but could not establish the dynamics of this decline. According to the same study, the number of other anterior pituitary cells did not change significantly during ageing. In the pituitary glands of individuals aged over 90 years, focal necrosis or scarring tissue, iron or amyloid deposits, basophilic invasions, accumulations of squamous cells, adenomas and granular cells were occasionally seen.

The results of our present immunohistomorphometric study of anterior pituitary glands, obtained from human male cadavers, indicated a significant increase in size of somatotropic cells with ageing, while the size of their nuclei remained unaffected by this physiological process. The increase in size of somatotropic cells was slow and steady, so statistically significant differences exist only between age groups I and III. On the other hand, nuclear-cytoplasmic ratio declined uniformly with years of age, given that statistically significant differences were observed only between age groups I and III. The increase in size of somatotropes during ageing, as shown in this study, is in accordance with the research of Antić et al. [20]. However, in contrast to these authors, we could not confirm any significant decline of volume density of somatotropic cells with advancing age. Our study showed that the appropriate structural changes in somatotropic cells are responsible for the functional decline of the GH / IGF-1 axis during human aging, which is consistent with the earlier work of Antić et al. [20]. The dynamics of these changes at the cellular level was influenced by numerous, insufficiently known, distant and local factors in the cell environment. It is certain that the reported age-related changes of somatotropes can be at least partly explained by structural changes at the level of hypothalamus occurring with advancing age (4).

Our results showed an increase in the volume density of FS cells in the elderly, compared to younger cases. This fact may indirectly indicate increased FS cell function in the elderly, as shown by Pavlović et al. [21] during examination of this parameter in the case of both sexes, when the significant increase was noticed after the age of 80. However, except for the fact that the male cases studied by Pavlović et al. [21] were older and with greater volume density of FS cells than in our study, this significant increase of volume density of FS cells was explained with simultaneous increases in the size and number of these cells in the pituitary mucoid wedge in their oldest group. Our histological analysis could not reveal any regional differences in the dynamics of FS cells during the process of ageing. From our results, we could make an

assumption that age-related increased volume density of FS cells is a two-phase process. The first phase, probably occurring in men aged 50 to 70 years, would predominantly be the consequence of increased size of FS cells or their hypertrophy, while in the second phase, occurring in men aged over 70 years, a further increase in size of FS cells occurs with a simultaneous increase of their number, leading thus to their significantly greater volume density. These facts may indirectly indicate increased function of FS cells in the elderly.

Analysis of the correlation between morphometric parameters of GH and FS cells indicated the presence of a statistically significant negative correlation between the average volume density of anterior pituitary FS cells and the nuclear-cytoplasmic ratio of GH cells, suggesting that homeostasis of growth hormone production/secretion exists [22].

CONCLUSION

From all the above, we concluded that in men, the size of GH cells increased with age. According to our results, it can be indirectly hypothesized that long-term hypertrophy of GH cells results in their functional decline after the age of 70. Furthermore, density and size of FS cells increased during ageing, which indicated their increased function. The strong correlation between morphometric parameters of FS and GH cells might point to age-related interactions between these two cell groups.

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Table 1. Morphometric analysis of immunoreactive growth hormone and folliculo-stellate cells of the anterior pituitary in all 14 analyzed cases

Case	Age	Group	V _{VGH} (%)	V _{VFS} (%)
1	41	I	19.87	1.59
2	45	I	15.20	1.16
3	48	I	19.56	1.88
4	48	I	21.43	1.60
5	57	II	11.71	1.33
6	61	II	19.72	2.94
7	65	II	25.35	3.44
8	65	II	26.35	2.46
9	66	II	19.65	2.26
10	76	III	28.32	2.26
11	76	III	22.69	2.81
12	77	III	19.35	3.13
13	78	III	13.42	3.37
14	87	III	22.74	4.79

V_{VGH} – volume density of growth hormone cells; V_{VFS} – volume density of folliculo-stellate cells

Table 2. Regression analysis between the age as predictor and area, nucleocytoplasmic ratio of somatotropes (GH) immunoreactive cells, as well as volume density of folliculo-stellate cells (FS) cells as outcome variables

A_{GH}					
Variable	B	SEB	β	t	p
Constant	94.00	18.53		5.07	<0.001
Age	0.97	0.28	0.70	3.41	0.01
$R^2 = 0.49$; $F(1,12) = 11.63$, $p=0.005$; Model: $A_{GH} = 94.00 + Age \times 0.97$					
$(N/C)_{GH}$					
Variable	B	SEB	β	t	p
Constant	0.35	0.04		8.57	<0.001
Age	-0.002	0.0006	-0.69	-3.32	0.06
$R^2 = 0.48$; $F(1,12) = 11.05$, $p=0.006$; Model: $(N/C)_{GH} = 0.352 - Age \times 0.002$					
V_{VFS}					
Variable	B	SEB	β	t	p
Constant	-1.06	0.78		-1.37	0.20
Age	0.06	0.01	0.80	4.62	0.001
$R^2 = 0.61$; $F(1,12) = 21.32$, $p=0.001$; Model: $V_{VFS} = Age \times 0.06 - 1.06$					

A_{GH} – area of GH cells; $(N/C)_{GH}$ – nuclear-cytoplasmic ratio of GH cells; V_{VFS} – volume density of folliculo-stellate cells; R^2 – the coefficient of determination; B – unstandardized coefficient; SEB – standard error of B; β – standardized coefficient beta; t – t-test

Table 3. Results of univariate ANOVA, testing between the average values of morphometric parameters of growth hormone immunoreactive and folliculo-stellate cells of the anterior pituitary in the analyzed age groups

Parameter	Group	N	Average	SD	SE	95% CI		Tukey post hoc test
						LB	UB	
A_{GH} (μm^2)	I	4	141.39	13.94	6.97	119.20	163.57	a
	II	5	150.55	21.18	9.47	124.26	176.85	/
	III	5	172.46	9.20	4.12	161.03	183.89	a
ANOVA	F(2,11) = 4.77, p = 0.03							
A_{NGH} (μm^2)	I	4	29.05	2.27	1.13	25.44	32.66	/
	II	5	26.99	5.27	2.36	20.45	33.53	/
	III	5	26.70	5.30	2.37	20.12	33.29	/
ANOVA	F(2,11) = 0.32, p = 0.73							
$(N/C)_{GH}$	I	4	0.26	0.02	0.01	0.23	0.29	a
	II	5	0.22	0.04	0.02	0.17	0.27	/
	III	5	0.18	0.04	0.02	0.14	0.23	a
ANOVA	F(2,11) = 6.38, p = 0.01							
V_{VGH} (%)	I	4	19.02	2.67	1.34	14.76	23.27	/
	II	5	20.55	5.84	2.61	13.31	27.80	/
	III	5	21.30	5.46	2.44	14.53	28.08	/
ANOVA	F(2,11) = 0.24, p = 0.79							
V_{VFS} (%)	I	4	1.56	0.30	0.15	1.08	2.03	a
	II	5	2.41	0.83	0.37	1.37	3.44	/
	III	5	3.21	0.94	0.42	2.04	4.38	a
ANOVA	F(2,11) = 5.08, p = 0.03							

a – I:III, p < 0.05

A_{GH} – area of growth hormone cells; A_{NGH} – area of growth hormone nuclei;

$(N/C)_{GH}$ – nuclear-cytoplasmic ratio of growth hormone cells; V_{VGH} – volume density of growth hormone cells; V_{VFS} – volume density of folliculo-stellate cells; SD – standard deviation; SE – standard error; LB – lower bound; UB – upper bound; CI – confidence interval

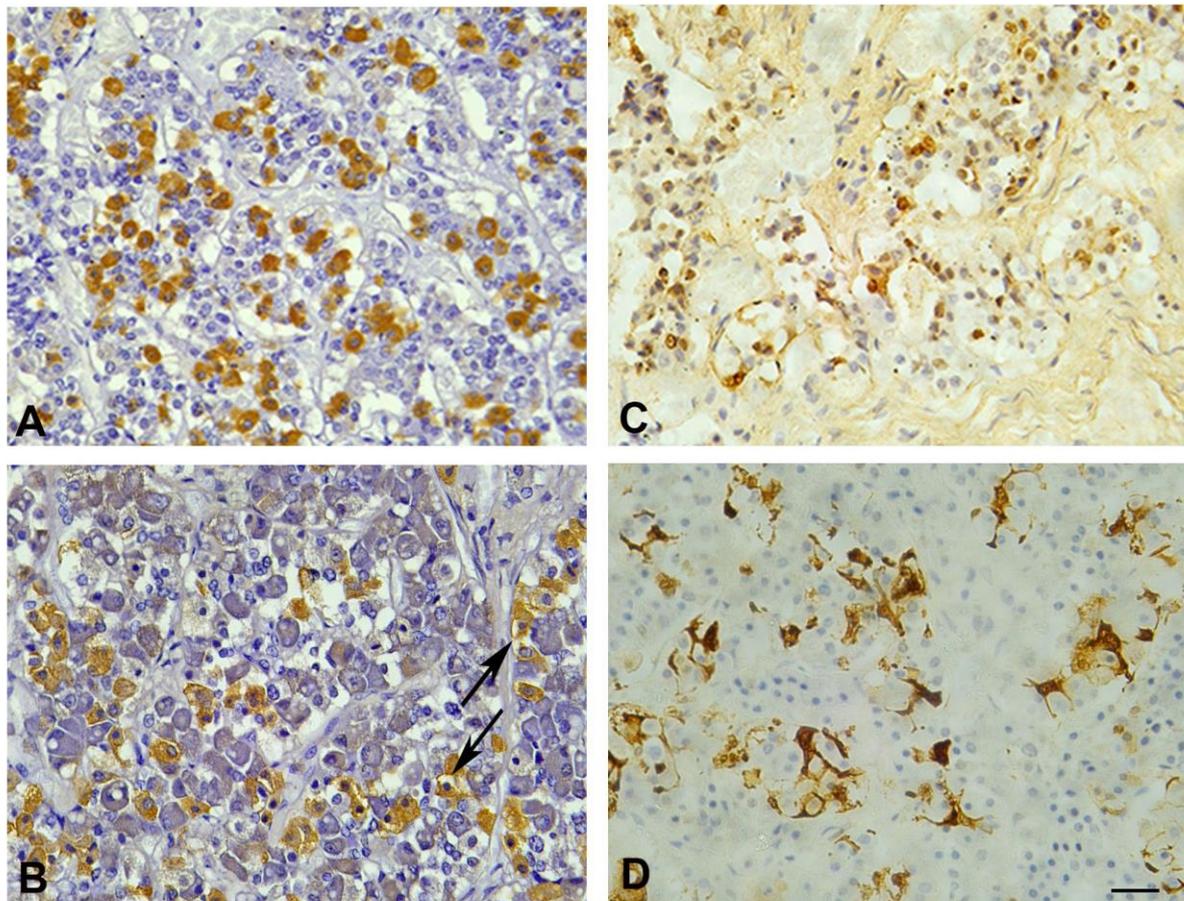


Figure 1. Representative micrographs of a young man's (41 years old) pituitary somatotrophic cells; A) older man's (87 years old) pituitary somatotrophic cells; B); S100 immunopositive folliculo-stellate cells in the 41- (C) and the 87-year-old man (D); Novocastra Peroxidase Detection System; magnification $\times 40$, bar = 30 μm

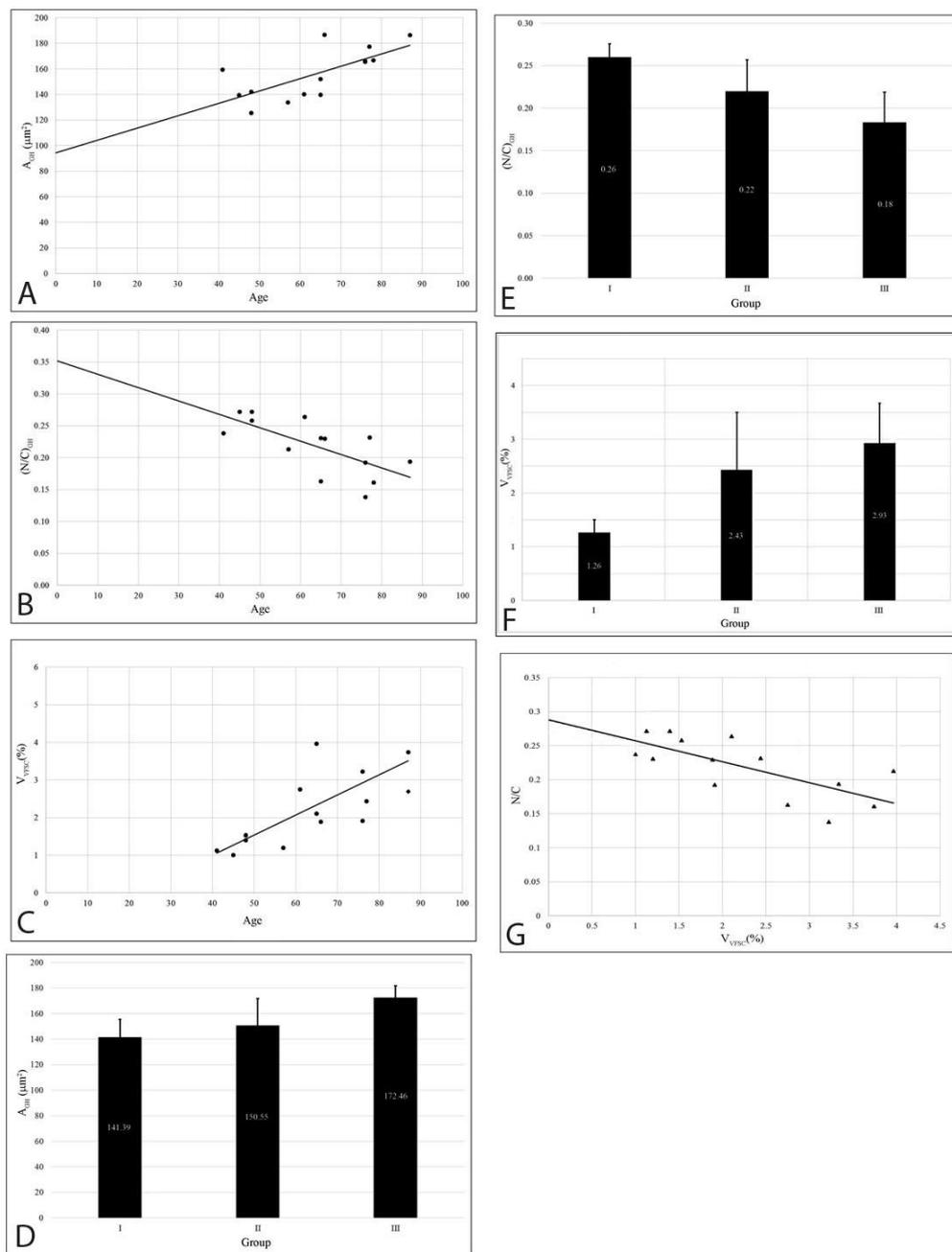


Figure 2. The graphical representation of the anterior pituitary gland parameters in the analyzed cases: correlation between age and area (A), nuclear-cytoplasmic ratio (B), average area (D), and average nuclear-cytoplasmic ratio (E) of growth hormone cells; correlation between age and volume density (C) and average volume density (F) of folliculo-stellate cells; correlation between volume density of folliculo-stellate cells and nuclear-cytoplasmic ratio of growth hormone cells (G);

V_{VGH} – volume density of growth hormone cells; V_{VFS} – volume density of folliculo-stellate cells; A_{GH} – area of growth hormone cells; $(\text{N/C})_{\text{GH}}$ – nuclear-cytoplasmic ratio of growth hormone cells

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