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Parahippocampal corpora amylacea and neuronal lipofuscin in human aging

Research Article

Mirjana D. Bakić[1], Ivan D. Jovanović[2]*, Slađana Z. Ugrenović[2], Ljiljana P. Vasović[2], Miljan S. Krstić[3], Natalija J. Stefanović[4], Miljana N. Pavlović[2], Vladimir S. Živković[2]

1 University of Podgorica, Medical Faculty, Department of Anatomy, Kruševac bb, 81000 Podgorica, Montenegro

2 University of Niš, Medical Faculty, Department of Anatomy, Blvd. Dr Zoran Djindjić 81, 18000 Niš, Serbia

3 University of Niš, Medical Faculty, Department of Pathology, Clinical Center Niš, Blvd. Dr Zoran Djindjić 48, 18000 Niš, Serbia

4 University of Niš, Faculty of Sport and Physical Education, Department of medicine, Čarnojevićeva 10A, 18000 Niš, Serbia

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Abstract: The aim of this research was to quantify the number of corpora amylacea and lipofuscin-bearing neurons in the parahippocampal region of the brain. Right parahippocampal gyrus specimens of 30 cadavers were used as material for histological and morphometric analyses. A combined Alcian Blue and Periodic Acid-Schiff technique was used for identification and quantification of corpora amylacea and lipofuscin-bearing neurons. Immunohistochemistry was performed using S100 polyclonal, neuron-specific enolase and glial fibrillary acidic protein monoclonal antibodies for differentiation of corpora amylacea and other spherical inclusions of the aging brain. Cluster analysis of obtained data showed the presence of three age groups (median age: I = 41.5, II = 68, III = 71.5). The second group was characterized by a significantly higher numerical density of subcortical corpora amylacea and number of lipofuscin-bearing neurons than other two groups. Values of the latter cited parameters in the third group were insignificantly higher than the first younger group. Linear regression showed that number of parahippocampal lipofuscin-bearing neurons significantly predicts numerical density of subcortical corpora amylacea. The above results suggest that more numerous parahippocampal region corpora amylacea and lipofuscin-bearing neurons in some older cases might represent signs of its' neurons quantitatively-altered metabolism.

Keywords: *Parahippocampal gyrus • Corpora amylacea • Lipofuscin • Aging • Morphometry*

1. Introduction

The parahippocampal region comprises the superficial allocortical and periallocortical areas which cover amygdala and hippocampal formation. Together these structures form the medial temporal lobe (MTL). It is composed of three different cortical areas: entorhinal, perirhinal and posterior parahippocampal [1]. Generally, it is now accepted that the MTL as a whole is somehow involved in the processing of memory. This function is enabled by its specific bidirectional connections with the cerebral cortex and the hippocampus [2,3].

Cognitive performance gradually decreases during normal brain aging [4]. Taking into the consideration the importance of the MTL in memory processing, a large number of cross-sectional volumetric neuroimaging studies were performed. They predominantly focused on the comparison of MTL structure volumes, or the

* *E-mail: ivanjov@sbb.rs*

degree of their atrophy, between healthy individuals, age-matched cases suffering from mild cognitive impairment (MCI) and cases at different stages of Alzheimer's disease (AD) [5-15]. Their results showed a significant reduction of MTL component volumes in cases with mild cognitive impairment and AD. This confirmed the position that the shrinkage of MTL volume is associated with memory decline, but whether this conclusion reflects the consequence of aging or not remains unclear. On the other hand, longitudinal studies performed by Raz and associates [16-19] used repeated measures models to focus on the dynamics of MTL component volumetric changes within the same individuals during the aging process. Their results varied from the general detection of minimal changes, or minimal till the age of 50, with an increased annual percentage of atrophy after the age of 50, up to the significant shrinkage of MTL structures, such as the entorhinal cortex.

These findings, and the fact that in the early phases of AD the first pathological changes occur in the entorhinal cortex [5-15], stressed the importance of the histological findings, especially of MTL areas, that might improve the understanding of the cellular basis for the various degrees of cognitive decline within healthy elderly populations. Until recently it was generally believed that age-related impairments in cognitive performance are the result of age-related neuron loss, especially within the hippocampus and most neocortical areas. However, with the development of more accurate stereological techniques, it emerged that there is limited neuron loss within the hippocampal region (hilus and subiculum) and that there is no evidence of a more extensive neuronal loss than 10% during normal brain aging in other brain regions [20], including the MTL [21-25].

These reports led Keller [4] to hypothesize that accrual of pathology during normal aging may play a contributing role in mediating the cognitive impairment observed within the elderly. He further states that the brain of healthy elderly individuals commonly harbors a number of pathological features, among which are AD-related pathologies, such as senile plaques and neurofibrillary tangles. The important fact to note is that in a certain number of these individuals, depending on which criteria are utilized, postmortem examination findings were sufficient for a diagnosis of likely or probable AD [26]. However, because the clinical manifestations of dementia in these subjects was not recorded during their lifetime, they cannot be diagnosed as being AD cases. This highlights the fact that there is a considerable discrepancy between the presence of AD-related pathology and the cognitive impairment observed in some individuals. Large numbers of pathological features, including corpora amylacea (CA), argyrophilic grains, neuromelanin and lipofuscin can be frequently observed in the aging brain and the brains of AD patients. This may lead to the conclusion that these can significantly influence the cognitive status of elderly individuals [4].

The term "corpora amylacea" (CA) is the most frequently used for certain polyglucosan bodies with a characteristic topography in the healthy nervous system of man and other species [27,28]. The role of CA has remained vague till recently Singhrao et al. [29,30] and Buervenich et al. [31] have emphasized the importance of CA as repositories, or a regulated disposal system, in the human brain of the products of neuronal cell death and myelin breakdown, both in aging individuals and in diseases, such as multiple sclerosis, AD and Parkinson's disease.

Melanin and lipofuscin are two pigments that can be found in the human brain. Both pigments have been until recently considered as inert waste products of neural tissue components of little interest to the neuroscience community. Lipofuscin is a protein and lipid-based pigment with broad distribution [32,33]. Another term found in the literature for lipofuscin is "ceroid". This term is used for disease-associated lipofuscin, which accumulates in disorders such as neuronal ceroid lipofuscinoses. Lipofuscin is formed exclusively within lysosomes. Consequently, lysosomal functions decrease over time and become incomplete. Its composition is variable in different cell types, but in neurons it is probably derived from autophagocytosed mitochondria and their incomplete digestion in lysosomes [34]. Once formed, lipofuscin cannot be degraded by lysosomal hydrolases and is also unable to be exocytosed. Accumulation of undigested cellular breakdown products into lipofuscin may benefit the cell in that potentially damaging metabolites are inactivated. However, the majority of studies have argued that accumulation of this pigment with aging is likely to be detrimental for neurons [32].

So a possible role of the parahippocampal region in the pathogenesis of cognitive decline in the elderly, and limited knowledge about the presence of CA and lipofuscin deposits in it as aging changes, led us to perform their quantification in this region of the brain in cases with different age.

2. Materials and methods

Tissue specimens from 30 cadavers (16 males and 14 females) were obtained from the rostal part of the right parahippocampal gyrus, no longer than 12 hours postmortem during routine autopsies performed at the Department of Forensic Medicine at Medical Faculty in Niš. According to the cadavers' medical documentation,

during their lifetime theyhave neither had a diagnosed brain injury nor any other neurological disorder. The age of cadavers ranged from 16 to 93 years. Tissue samples were fixed in 10% buffered formalin, embedded in paraffin and cut into sections 5 μm thick. Tissue sections were processed by routine histological procedures and stained using a combined Alcian Blue and Periodic Acid-Schiff technique (AB PAS), because this best enabled simultaneous detection of subpial and subcortical CA and neuronal lipofuscin deposits in laminae II – V of the parahippocampal gyrus cortex in each of the 30 evaluated cases. Sections of 12 cases were additionally immunohistochemically stained (Dako, APAAP kit) using a neuron specific enolase (NSE) monoclonal antibody (Dako, code no. M0873), S100 polyclonal antibody (Dako, code no. Z0311) and glial fibrillary acidic protein (GFAP) monoclonal antibody (Dako, code no. M0761). Sections of the parahippocampal tissue stained only with secondary antibody served as negative control. All stained histological slices were analyzed using light microscopy (Nikon Eclipse E100) under 40x and 100x magnification. AB PAS stained sections were used for morphometric analysis. Digital images were captured using a digital camera (AmScope MU Series) and its software (AmScope ToupView, version 3.2.1476) under 40x magnification. The whole subpial region of the parahippocampal gyrus from each of the 30 analyzed cases was subdivided into ten regions, and from each of these regions one field of vision, with the same X and Y coordinates, was captured under 40x magnification for the morphometric analysis of subpial CA [35]. Under the same magnification, this method was used to select and capture 10 additional fields of vision from the subcortical region of the parahippocampal gyrus for the morphometric analysis of subcortical CA and, and a further 10 fields of vision from lamina II – V from each case for the analysis of the presence of lipofuscin-bearing neurons.

Morphometric analysis was performed using ImageJ image processing and analysis software. It included measurement of subpial CA numerical density (N_{VSP}) and Feret's diameter (D_{FSP}), subcortical CA numerical density (N_{VSC}) and Feret's diameter (D_{FSC}), and the number of lipofuscin-positive (N_{AL}+) and lipofuscin-negative (N_{AL}-) neuronal profiles in the test area. The counting of CA, lipofuscin-positive and lipofuscin-negative neurons was performed using the cell counter plugin. Feret's diameter was measured for all observed CA. The test area for the measurement of subpial CA numerical density was the superficial layer of the parahippocampal gyrus. Its area was measured using a square line grid (a = 326.66 μm^2) which was overlaid on digital images, and the number of test points which hit this layer of the parahippocampal gyrus cortex were counted using the cell counter plugin. The test area was obtained as product of the number of these points and the area of one test point (a). The test area for the subcortical CA numerical density and the number of lipofuscin-positive and lipofuscin-negative neurons was the area of the whole image. Numerical density and number of lipofuscin-positive and lipofuscin-negative neurons in the test area was calculated according to the formulae given by Kališnik et al. [36].

3. Statistical methods

Statistical analysis was performed with NCSS – PASS 2007 statistical package. Classification of cases was performed by cluster analysis (k – means method) during which age, N_{VSP}, N_{VSC} and N_{AL}+ were used as classification parameters. Three or more median values of morphometric parameters were compared using a Kruskal – Wallis non-parametric test with a post hoc analysis using Dunn's z test. Median values of two independent groups were compared using the Mann – Whitney U test, while median values of two dependent groups were compared by the Wilcoxon Signed-Rank test. Correlation analysis was performed by Spearman's rho (ρ) calculation. The relationship between the number of lipofuscin-positive neurons in the test area as a predictor, and the numerical density of subcortical CA as the outcome variable, was evaluated by linear regression analysis.

4. Results

4.1. Morphological analysis

Histologically, CA were identified in the subpial region and subcortical white matter of the parahyppocampal gyrus. They were purple or dark blue on AB PAS stained sections (Figure 1A – D). Younger cases, and a number of older cases, contained sparse subpial and subcortical CA. In such cases, rare CA were mostly localized immediately beneath the pia mater in the subpial region (Figure 1A), while in the subcortical white matter, they were observed scattered between the oligodendrocytic nuclei and next to the subcortical blood vessels (Figure 1B). The rest of the elderly cases contained more numerous CA. In the subpial region, they were observed beneath the pia, as well as in the deeper parts of the molecular layer. The "yeast" form of CA, characterized by more densely-stained cores than periphery and radial striations between them, were seen more frequently in these zones of the parahippocampal gyrus cortex than

in younger cases (Figure 1C). In the subcortical white matter, a large number of CA occupied the space around the arteriolar vessels with thickened walls (Figure 1D). In cases in which they occupied the space around the venular vessels, their lumina were collapsed.

Laminae II – V of the younger cases' parahippocampal gyrus cortexes contained neurons of different size and shape, perineuronal oligodendrocytes and astrocytic nuclei. On AB PAS stained sections, contours of the neuronal bodies were observed with the large euchromatic nuclei and prominent eccentric nucleoli inside them. Narrow band of perikarya surrounded neuronal nuclei. These laminae of some older cases' parahippocampal gyruses were characterized by the presence of a more pronounced perineuronal halo and occasionally more numerous oligodendrocyte satellite cells, though the perineuronal halo might be the consequence of the tissue processing procedure. Prominent red-stained granular lipofuscin deposits were observed in neuronal perikarya. Older cases with more numerous CA were characterized by more frequent lipofuscin-bearing neurons. In such cases neuronal lipofuscin deposits were extremely large and consequently caused peripheral displacement of their nuclei (Figure 1E). Neurons of the older cases, in which CA were less frequent, contained smaller lipofuscin cytoplasmic deposits which did not disturb their morphology (Figure 1F).

Application of the NSE monoclonal antibody showed a positive reaction across the whole area of CA profiles. A positive reaction with a similar intensity of the surrounding neuropil and neuronal perikarya served as an internal positive control, but made more detailed analyses of CA immunoreactivity difficult (Figure 2A). A similar reaction was observed after the application of S100 polyclonal antibody. Corpora amylacea with a positive reaction across their whole area, as well as the ones with a stronger reaction at their periphery and a weaker in their core, were observed in the S100 positive neuropil (Figure 2B). A positive reaction of the surrounding glial cells served in this case as an internal positive control. The immunohistochemical reaction of observed structures on these two antibodies pointed to the conclusion that they probably contained both neuronal and glial components. The reaction of CA on application of the monoclonal antibody for glial fibrillary acidic protein (GFAP) was predominantly negative (Figure 2C). They were mostly observed as hollow spaces in the neuropil of the parahippocampal gyrus, sometimes surrounded by the narrow GFAP-positive zones and GFAP-positive astrocytes (internal positive control) in the subcortical white matter, around the blood vessels.

The results of the morphological and immunohistochemical analyses differentiated CA from other similar spherical inclusions that might be present in the nervous tissue in the elderly cases.

Results of the morphometric analysis are presented in Table 1. A linear correlation analysis did not establish the presence of a significant correlation between age and any of the measured morphometric parameters (Table 2). A cluster analysis was further performed in order to evaluate the presence of other types of relationship between the age and measured morphometric parameters. It revealed that there were three groups of cases with significantly different numbers of CA and lipofuscin-bearing neurons (Table 3). Their ages significantly differed ($H=13.96$, $DF=2$, $p=0.0009$). The median age of the first group was 41.5 years. The second group had a median age of 68 yearswhich was significantly higher than the age of the first group ($p<0.01$). The third group's median age was 71.5 years and was significantly higher than the age of the first group ($p<0.01$). The age of this group was higher than the age of the second group, but this difference was not significant ($p>0.05$).

The median values of all CA morphometric parameters showed a similar trend. They had the lowest values in the first and the highest in the second group. Values of the third group were lower than the second, but higher than the first group (Table 3). However, these differences were significant only in the case of subcortical CA numerical density ($H=19.87$, $DF=2$, $p=0.00005$) and Feret's diameter ($H=6.6$, $DF=2$, $p=0.037$). The numerical density and Feret's diameter of subpial CA of the established groups were not significantly different ($p>0.05$). Cases of the second group were characterized by more numerous and larger subcortical CA. This confirmed a significantly higher median numerical density of this group's CA than that of the first and the third groups' ($p<0.01$) (Figure 3A). The value of the second group's Feret's diameter was significantly higher than that of the first group's CA ($p<0.01$). It was also higher than the third group's CA, but this difference was not significant ($p>0.05$) (Figure 3B). Additionally, the median numerical density of the subcortical CA was significantly higher than the subpial ones in the first, and especially in the second, groups ($z= -1.83$, $p= 0.034$; $z=2.66$, $p=0.008$, respectively) (Figure 3A). Moreover, an analysis of gender differences (Table 4) showed that the median numerical density of subcortical CA was significantly higher in females than in males ($z=2.02$, $p=0.04$) (Figure 3C). Also, the median numerical density of subcortical CA in female cases was significantly higher than that of subpial CA ($z=2.67$, $p=0.008$) (Figure 3C).

The median number of lipofuscin-bearing neurons followed the trend of CA (Table 3). It was significantly different in the obtained groups ($H=20.15$, $DF=2$, $p=0.00004$), with the second group value being

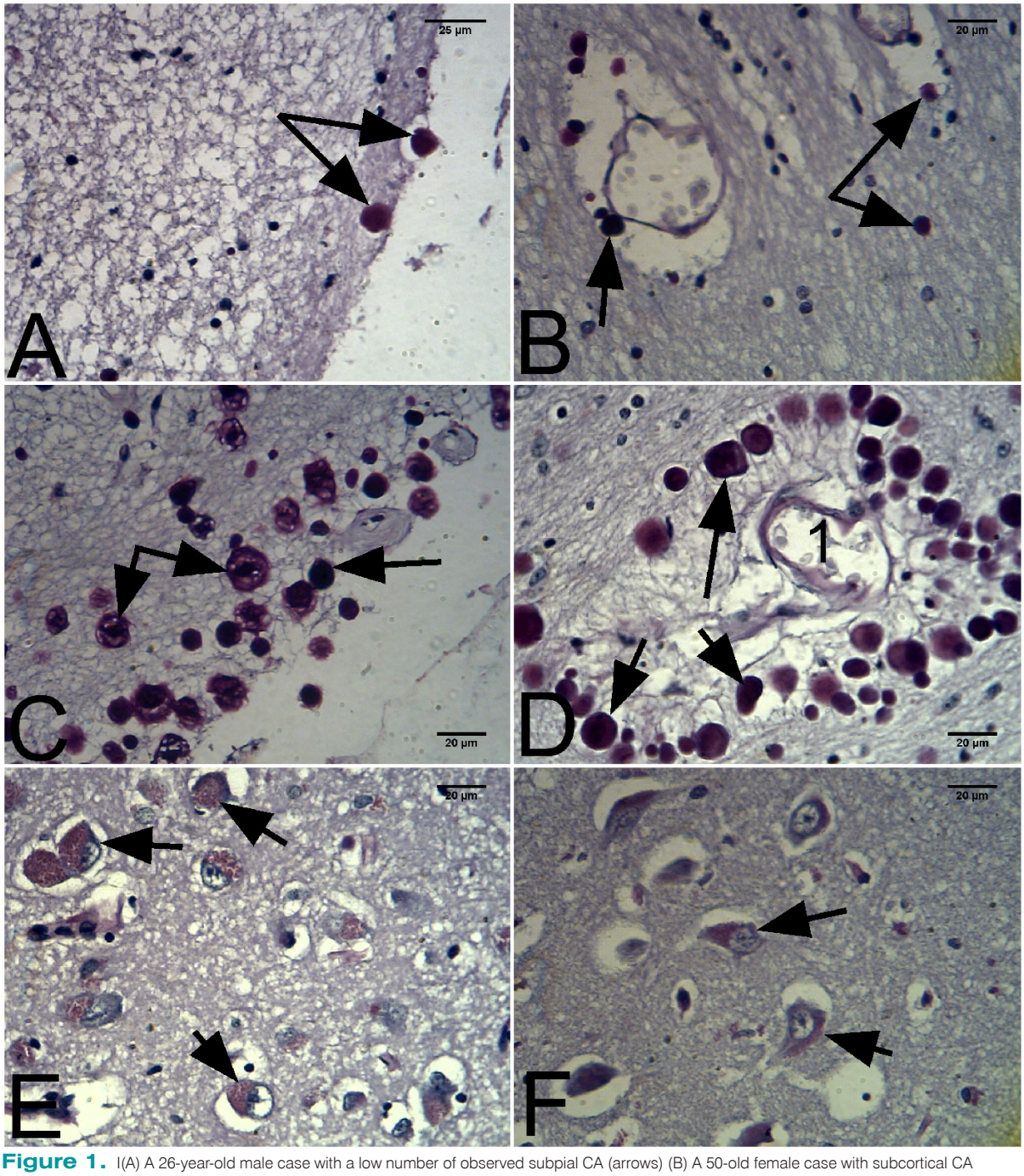

Figure 1. I(A) A 26-year-old male case with a low number of observed subpial CA (arrows) (B) A 50-old female case with subcortical CA (arrows) (C) An 84-year-old male case with the yeast form of subpial CA (arrows) (D) A 70-year=old female case with subcortical CA (arrows); 1 – subcortical blood vessel (E) Neurons with significant lipofuscin inclusions of a 78-yearold male case (arrows) (F) A 65-year-old female case with less frequent lipofuscin-positive neurons and smaller lipofuscin deposits in their perikarya (arrows); AB PAS; 40x lens magnification.

significantly higher than the ones in the first and the third group ($p<0.01$). Its value in the third group was higher than in the first, but this difference was not significant ($p>0.05$) (Figure 3D). However, the median number of lipofuscin-positive neurons was significantly higher than the ones without lipofuscin deposits only in the second group ($z=2.20$, $p=0.028$). In the first group it was significantly lower than the number of the neurons without lipofuscin deposits ($z=2.52$, $p=0.01$), as it was in the third group ($z=1.89$, $p=0.03$).

Gender differences in the number of lipofuscin-bearing neurons were present, too (Table 4). It was

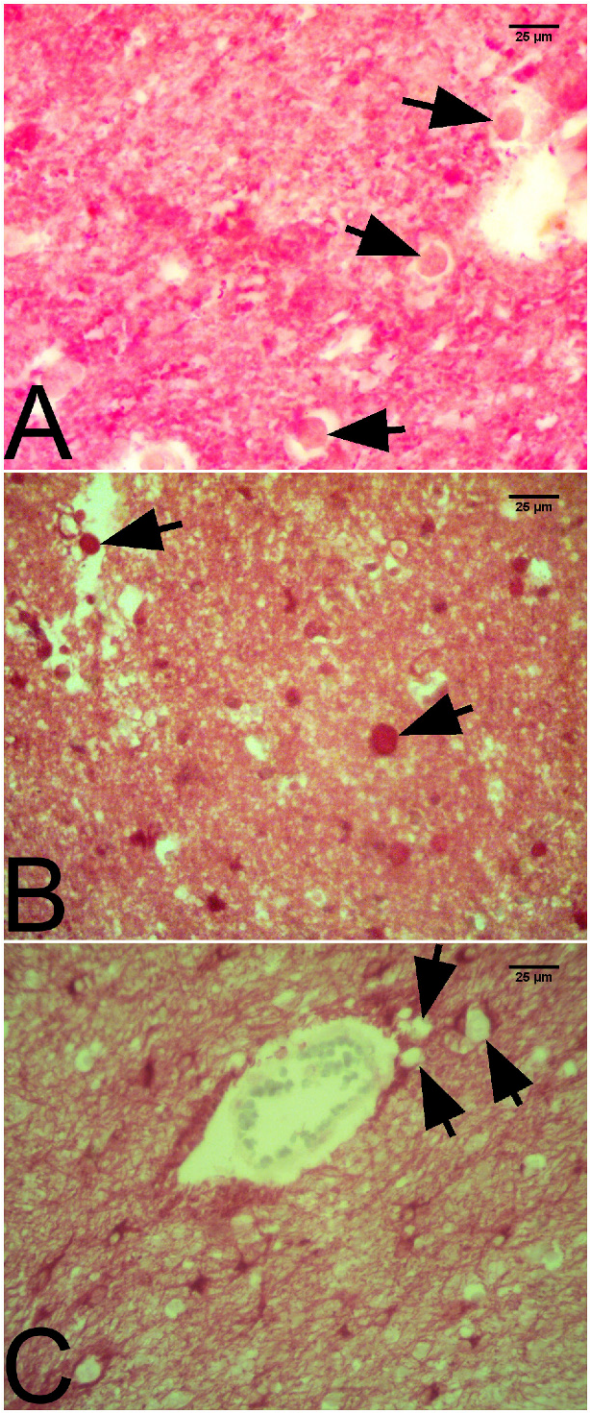

Figure 2. (A) A 57-year-old male case with NSE-positive subcortical CA (arrows) (B) A 70-year-old male case with S100 positive subcortical CA (arrows) (C) A 58-year-old female case with GFAP-negative CA (arrows); APAAP; 40x lens magnification.

significantly higher in female than in male cases (z=1.66, p=0.048) (Figure 3E). Furthermore, the number of lipofuscin-free neurons was significantly higher than lipofuscin-positive neurons in males (z=1.84, p=0.033), while in female cases these values were not significantly different (p>0.05) (Figure 3E).

The linear correlation analysis (Table 2) showed that a significant positive correlation existed between the numerical density of subcortical CA and the number of lipofuscin-bearing neurons (ρ=0.83, n=23, p<0.0001). Additionally, linear regression analysis showed that the number of lipofuscin-bearing neurons significantly predicts the numerical density of subcortical CA (F(1,26)=18.44, p=0.0002) and explains 39% of its variance (adjusted R square = 0.39) which represents a large effect (Figure 3F).

5. Discussion

According to Leel-Ossy [37], in the cerebrum CA might be detected in very large numbers beneath the ependymal lining of the roof and the floor of the third and fourth ventricles, and in the roof of the cerebral aqueduct. On the outer surfaces of the brain they are most often observed in the glial feltwork beneath the pia, where they lie especially in the depths of sulci and in the outer part of layer I of the cortex. In our case, CA were observed immediately beneath the pia mater in younger cases, while a significant number in the outer part of the parahippocampal gyrus cortex layer I was detected only in older cases. When present in the white matter, CA tend to congregate around vessels of medium and large size in the Virchow–Robin spaces, which was observed during our research, too. Their mean diameter is regularly 10–12 µm [27], or according to Buervenich et al. [31] 16 µm. These are in accordance with our results. Concentric laminated or target forms, which have more densely-stained cores than peripheries, similar to true Lafora Bodies, might occur more or less frequently [27, 31]. In our study they were observed in a few older cases, mostly in the subpial region of the parahippocampal gyrus.

Histochemically, these round deposits may be clearly detected by conventional stains such as hematoxylin-eosin, alcian blue, aldehyde fucsin and periodic acid Schiff, which reveal the existence of acidic and neutral mucopolysaccharides and oligosaccharides in their structure [38]. All CA stain strongly with cresyl violet [31]. The same authors detected a good reactivity of CA on GS-I lectin and concluded that this reaction could connect them with the degenerative changes in the brain [38].

Immunohistochemical reactions indicate that CA probably contain materials derived from several

Table 1. Morphometric characteristics of the CA and lipofuscin-bearing neurons in the parahippocampal gyrus cortex of all evaluated cases

Case	Case	Age	Gender	Group	N_{Vsp} (mm^{-3})	D_{Fsp} (μm)	N_{Vsc} (mm^{-3})	D_{FSC} (μm)	N_{aL}- (mm^{-2})	N_{aL}+ (mm^{-2})
12	1	16	male	I	0.43	15.34	0.70	8.95	203.34	37.54
29	2	26	male	II	52.79	14.36	47.5	14.88	140.77	156.41
6	3	37	male	I			10.37	6.64	140.77	28.15
1	4	38	male	I	0.45	6.64	0.45	6.88	143.9	18.77
14	5	40	male	I					184.57	31.28
27	6	43	male	I	11.95	9.98			269.03	75.08
3	7	50	female	I	4.45	8.30	8.59	10.19	131.39	100.1
25	8	54	male	II	0.61	9.40	38.65	10.68	156.41	131.39
4	9	57	male	I	0	0	5.24	10.74	162.67	71.95
7	10	58	female	III	20.88	10.72	41.29	9.32	71.95	71.95
13	11	61	male	I	0.76	12.93	3.03	12.41	145.04	54.03
23	12	61	female	II	34.20	12.16	61.57	12.35	128.26	115.75
26	13	61	female	II	5.39	10.69	94.72	11.43	84.46	153.28
21	14	63	female	II	31.77	11.54	51.02	11.71	93.85	156.41
17	15	64	male	III			3.03	12.40	100.1	150.16
2	16	65	female	III	36.23	10.32	30.39	10.81	100.1	84.46
9	17	65	female	III			3.16	13.88	122	118.87
11	18	67	female	II	0.7	14	45.80	11.95	56.31	115.75
24	19	69	female	II	7.60	11.89	65.59	11.59	137.64	115.75
10	20	70	male	III	1.14	15.91	28.16	10.78	122	100.1
18	21	70	female	II			70.29	13.35	100.1	122
15	22	71	female	II	0.56	11.4	49.01	11.94	78.21	128.26
5	23	73	male	III	0.97	9.11	1.55	8.06	96.98	65.69
16	24	75	male	III	0.25	12.81	15.5	12.92	118.87	112.62
28	25	78	male	II	85.63	13.52	67.85	11.48	90.72	259.65
20	26	80	male	II	3.13	10.14	66.85	12.96	79.63	119.44
8	27	84	male	III	77.58	13.47	1.91	9.84	100.1	71.95
30	28	84	female	III	0.27	11.17	22.77	8.66	237.75	90.72
19	29	86	female	III	9.21	9.34	0.22	14.10	172.05	78.21
22	30	93	female	II	31.31	11.97	52.41	12.77	109.49	128.26

N_{VSP}–subpial CA numerical density
D_{FSP}–subpial CA Feret's diameter
N_{VSC}–subcortical CA numerical density
D_{FSC} – subcortical CA Feret's diameter
N_{AL+}–number of lipofuscin-positive neuronal profiles in the test area
N_{AL-}–number of lipofuscin-negative neuronal profiles in the test area

sources. Positive reactions to anti-tau antibodies, the extracytoplasmic domain of amyloid precursor protein (APP), serum carnosinase, α-synuclein, anti-NSE, anti-NeuN and anti-nestin antibodies suggest a neuronal source for some constituents [27,31,39]. The results of a proteomic analysis by Selmaj et al. [40] provided evidence that the biogenesis of CA involves degeneration of cells of neuronal origin. Corpora amylacea also react with antibodies to advanced glycation end-products, which in nervous tissue are also found in neuronal lipofuscin granules [41]. The presence of cell stress proteins, such as ubiquitin, ubiquitin related PGP9.5, heme oxygenase-1 and heat shock proteins 28, 32, 60, 70 and 72, could originate from both neurons and glia [27,31]. Positive reactions of CA to antibodies to myelin basic protein, proteolipid protein, galactocerebroside, myelin/oligodendrocyte glycoprotein and to antiferritin antibodies indicate an oligodendroglial contribution to their composition [29,31]. Conversely, CA normally always displayed GFAP-negativity inside their cores, while GFAP-positivity was observed only in the border zone or "capsule" of the CA [29,31,37], like in our case. CA were unreactive with microglial monoclonal MRC-OX42 antibody [29]. Anti-vimentin antibody and anti-neurofilament antibody did not label any of the observed CA [31]. Singhrao et al. [30] found positive reactions

Table 2. Correlation matrix between the age, numerical density of subpial CA, numerical density of subcortical CA, number of lipofuscin-bearing and number of lipofuscin-free neurons.

Spearman Correlations Section (Row-Wise Deletion)					
		N_{Vsp}	N_{Vsc}	N_{aL}-	N_{aL}+
Age	ρ	0.12	0.09	-0.21	0.10
	p	0.60	0.67	0.35	0.64
	n	23	23	23	23
NVsp	ρ		0.40	-0.28	0.35
	p		0.06	0.19	0.10
	n		23	23	23
NVsc	ρ			-0.53	0.83
	p			0.009	0.000
	n			23	23
NaL-	ρ				-0.35
	p				0.10
	n				23

Table 3. Morphometric characteristics of CA and lipofuscin-bearing neurons in groups obtained as the result of cluster analysis.

		Group		
Variables	Parameter	I (n=8)	II (n=12)	III (n=10)
Age	Mean	42.75	66.08	72.40
	Median	42	68	71.5
	SE	4.94	4.71	3.08
N_{Vsp} (mm^{-3})	Mean	3.61	23.06	18.32
	Median	0.76	7.60	5.17
	SE	2.22	8.27	9.61
D_{Fsp} (μm)	Mean	10.64	11.91	11.61
	Median	9.98	11.89	10.95
	SE	1.57	0.47	0.82
N_{Vsc} (mm^{-3})	Mean	4.73	59.27	14.80
	Median	4.13	56.99	9.33
	SE	1.68	4.37	4.73
D_{FSC} (μm)	Mean	9.30	12.26	11.08
	Median	9.57	11.94	10.79
	SE	0.92	0.32	0.68
N_{aL}- (mm^{-2})	Mean	172.59	104.65	124.19
	Median	153.85	96.98	109.49
	SE	16.25	8.73	15.07
N_{aL}+ (mm^{-2})	Mean	52.11	141.86	94.47
	Median	45.79	128.26	87.59
	SE	9.96	11.63	8.35

of CA for both classical and terminal pathway-specific components in tissues from MS patients, while normal

Table 4. Morphometric characteristics of CA and lipofuscin-bearing neurons in male and female cases.

Variables	Parameter	Female	Male
Age	Mean	68.79	56.00
	Median	66	59
	SE	3.12	5.16
N_{Vsp} (mm^{-3})	Mean	15.21	19.64
	Median	8.40	1.06
	SE	4.19	9.40
D_{Fsp} (μm)	Mean	11.12	11.97
	Median	11.28	12.87
	SE	0.42	0.82
N_{Vsc} (mm^{-3})	Mean	42.63	20.77
	Median	47.40	7.80
	SE	7.28	6.62
D_{FSC} (μm)	Mean	11.72	10.69
	Median	11.82	10.76
	SE	0.43	0.65
N_{aL}- (mm^{-2})	Mean	115.97	140.93
	Median	104.80	140.77
	SE	12.36	12.13
N_{aL}+ (mm^{-2})	Mean	112.84	92.76
	Median	115.75	73.51
	SE	6.83	15.51

brain tissue showed either less strong or negative responses. Hoyaux et al. [41] and Buervenich et al. [31] demonstrated that CA also contain a number of calcium-binding proteins (S100A1, S100A2, S100A3, S100A4, S100A5, S100A6, S100A8, S100A9 and S100A12). S100B protein, which is abundant in astrocytes, and to a lesser extent in neurons from normal brains, was not detected in CA [41]. Positive reactions of the structures observed during our research on monoclonal anti–NSE antibody and polyclonal S100 protein antibody, the predominantly negative reaction on monoclonal anti–GFAP antibody, together with their reaction on classical AB PAS staining are in agreement with above described reactions of CA.

As far as topography concerns, CA generally may be found in elderly subjects in almost all regions of the brain, but are notably concentrated in certain locations like the base of the brain, on the medial surfaces of the temporal lobes and over the hippocampal formations [27,42]. However, their presence in MTL structures was focused predominantly in the hippocampal formation, especially during some pathological conditions [43].

In spite of this mélange in the research concerning the structure and role of CA, we could not find any data about the dynamics of their presence in the parahippocampal gyrus during the aging process, considering its

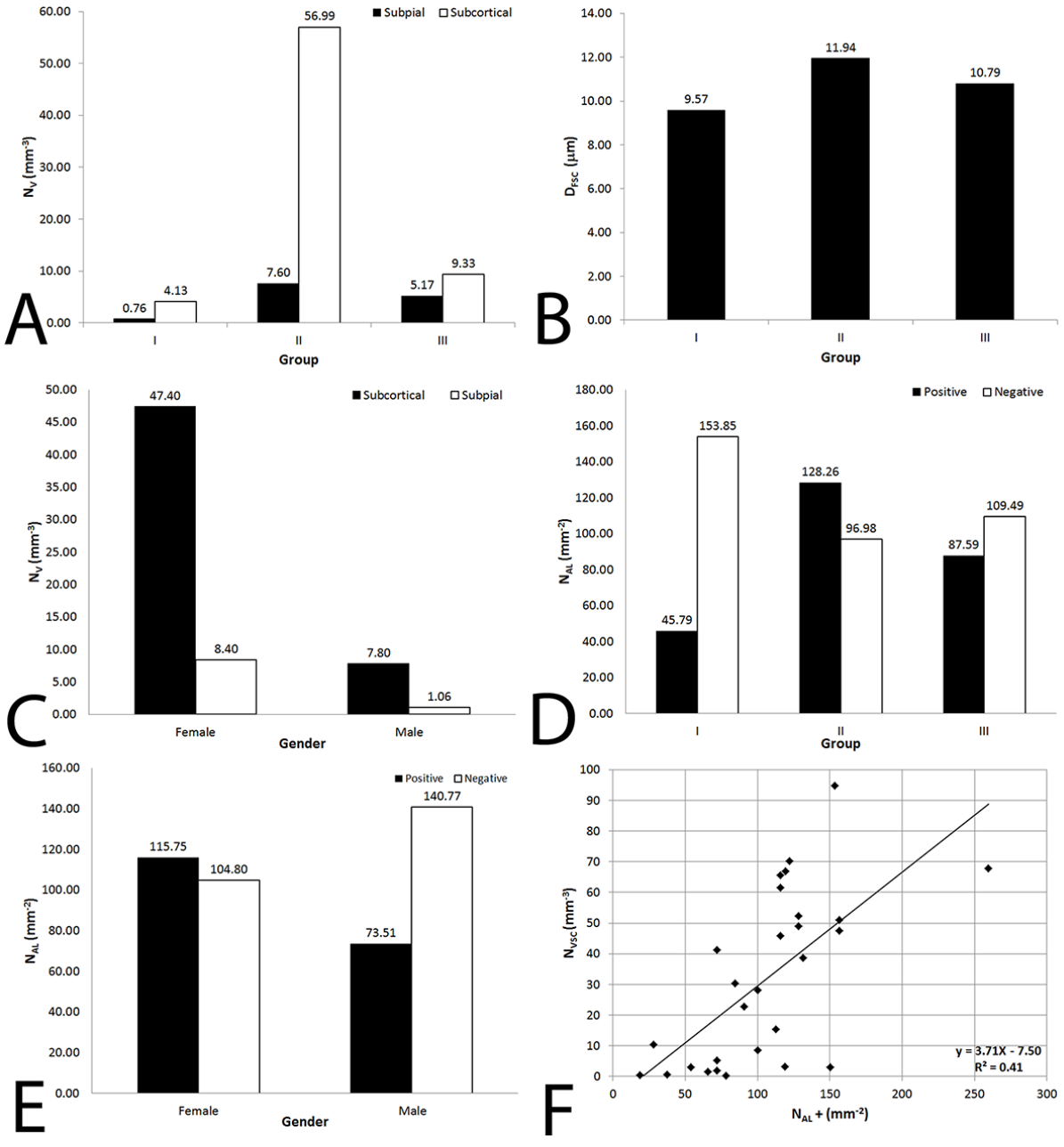

Figure 3. (A) Numerical density of subpial and subcortical CA in groups obtained during cluster analysis; (B) Feret's diameter of subcortical CA in the same groups (C) Numerical density of subpial and subcortical CA in male and female cases (D) Number of lipofuscin-bearing and number of lipofuscin free neurons in the groups obtained during cluster analysis (E) Number of lipofuscin-bearing and lipofuscin-free neurons in male and female cases (F) Scatter plot with regression line of the number of lipofuscin-bearing neurons as a predictor and numerical density of subcortical CA as outcome variable.

functional importance and role as a part of the MTL for the normal memory process. However, Abel et al. [39] presented the case of a 49-year-old woman where pathological examination of the anterior and mediotemporal lobe revealed diffuse and focally-dense white matter CA. This CA deposition was highly concentrated in the mediotemporal lobe and parahippocampal white matter, while CA were not seen in cortical gray matter, which is in agreement with our observations. Such pathological accumulation of CA in the parahippocampal white matter has been explained as an atypical presentation of adult polyglucosan body disease (APBD), but the influence of aging was not excluded [39]. Subjectively, this case suffered from some forms of changes in cognition.

All this might indirectly indicate that massive accumulation of CA in human parahippocampal gyrus can cause cognitive disturbances in such individuals. Cases of the second group obtained as the result of our cluster analysis were characterized by a similar CA deposition, but in their medical documentation there were no data regarding their cognitive status during the lifetime, and the effect of such CA deposition and simultaneous lipofuscin accumulation on their cognitive status could not be confirmed. However, the possible effects of such an accumulation of CA, especially in the subcortical region of the parahippocampal gyrus, on its metabolic and subsequent functional status cannot be excluded. The fact that subcortical CA were more numerous and larger in females than in males in our research is in agreement with the findings of Abel and associates [39].

There are little systematic quantitative data to support the generally held view that the number of CA increase with age. Cavanagh [27] cited that CA are almost invariably less frequent in younger age groups. The mean numbers of such bodies per microscope field in frontal and temporal cortical grey matter are exceedingly variable, regardless of sex or age. Generally, after the age of 30–40 years, CA become larger and increasingly numerous, although their counts per section vary strikingly and there are cases over 75 years old that still have number of CA as low as the 30 year olds. We observed an increase in the number of subpial, and especially subcortical, CA with age in our sample. However, opposite to the second group, in which this increase was significant in relation to the first, younger group, the number of CA of the third and the oldest group was insignificantly higher than that of the first and the youngest group in our case. This is in agreement with latter cited data [27].

Under light microscopy, lipofuscin appears yellow-brown or translucent and is histochemically characterized by sudanophilia, argyrophilia and periodic acid-Schiff positivity [44]. Lipofuscin granules are mainly found in the perinuclear area, as we observed, but may occasionally be present in other parts of the perikaryon. They can also be found in dendrites, axons and even presynaptic areas. Furthermore, lipofuscin is considered to be a ubiquitous pigment within the brain and its amount correlates with neuronal age. It is produced in nearly all cells of the human brain [32]. However, Double and associates [32] described that pyramidal cells within the aged human cortex contain fine grains of lipofuscin scattered throughout the cell body, while non-pyramidal neurons contain either larger lipofuscin granules or none at all. The lipofuscin deposits detected during our research were composed of fine, red-stained granules, localized in perikarya of the parahippocampal region cortical neurons, similar to those described by Double et al. [32] in pyramidal neurons. So, taking into the consideration the above cited facts and the knowledge about the structure of parahippocampal gyrus cortex, especially its entorhinal region, it can be concluded that the lipofuscin deposits observed during our study were probably the most frequently localized in the perikarya of its pyramidal neurons. Neuronal lipopfuscin deposits could coexist with large lipopigment storage vacuoles in all types of glial cells. Glial systems play an important role in collecting neuronal lipopigment, clusters of which especially deposit in pericapillary areas [32,45], or in areas where we observed numerous subcortical CA of the parahippocampal gyrus cortex.

Lipofuscin formation begins in early childhood and it accumulates slowly over time. By the third decade of life, most cortical neurons contain lipofuscin and the number of granules within individual cells becomes more abundant [32,44]. As far as the medial temporal region is concerned, Nakano et al. [46] described that accumulation of lipofuscin starts in the rat after sexual maturation and the region of the brain in which these deposits start to accumulate is the hippocampus. However, none of the latter cited authors do not mention the presence of such deposits in the human parahippocampal region of the cerebrum. Shimada et al. [47] discovered cytoplasmic ubiquitinated inclusions in neurons of SAMP10 mouse brains that appeared in an age-dependent manner. These inclusions were pale and grey-brownish in color and were not highlighted with HE stain. PAS stain highlighted them as clusters of dot-like structures, while fluorescence and confocal laser scanning microscopies revealed that most of their parts had autofluorescent properties. PAS-positive dot-like and autofluorescent features were consistent with classical lipofuscin granules. Inclusion-bearing neurons were densely distributed in the limbic system and related forebrain structures. Layer II neurons of the piriform and entorhinal cortices, and the neocortex layer V neurons were most affected by ubiquitinated inclusions. Piriform and entorhinal cortices are significant parts of the parahippocampal gyrus cortex and the localization of such inclusions, which are closely associated with lipofuscin granules, are in accordance with our findings of increased lipofuscin accumulation in parahippocampal neurons, especially in the second group obtained as a result of cluster analysis. Shimada et al. [47] further described that neurons bearing inclusions were degenerated in terms of having retracted dendrites, and the functional significance of neuronal degeneration in the limbic structures can be related to age-related behavioral impairments in SAMP10 mice. This might support our assumption that in the second group cases, the frequent

presence and predomination of the lipofuscin-bearing neurons in relation to the lipofuscin-free ones, as well as a significantly higher CA presence, might represent the signs of the some kind of metabolic dysfunction of the neurons in this region of the brain during their lifetime.

Neuronal lipofuscin may occupy up to 75% of the perikaryon. Modest quantities of lipofuscin, like the ones detected in the third group during our research, may not have discernible effects upon the cell. Heavy lipofuscin load, like the deposits observed in the second group cases in our study, induced by advanced age may compromise normal cellular functions [32]. Lipofuscins storage is correlated with the appearance and development of cytoskeletal damage, as well as with amyloid deposit and amyloid-related pathologic conditions [45]. In this light, lipofuscin cannot be seen as a benign entity, but must be recognized as a clear threat to homeostasis in postmitotic cells. Based on its prevalence in motor areas and the cerebral cortex, it might be assumed that it is involved in the changes in motor fidelity and cognitive decline that accompany aging. This hypothesis is predicated on the existence of a deleterious influence of lipofuscin accumulation during the inherited, fatal neurological disorders known as the neuronal ceroid lipofuscinoses [33,44].

A significant positive correlation between the number of lipofuscin-bearing neurons and number of CA established during our research impose a dilemma: does it merely represent an epiphenomenon induced by aging or does it reveal possible common mechanisms that underlie the formation of both cited cellular inclusions?" Kimura et al. [48] in their immunohistochemical study identified CML and pentosidine in certain aging-related brain inclusions, such as lipofuscin granules and CA. CML and pentosidine are considered to be glycoxidation products, and the formation of these products might reflect direct oxidative damage *in situ*, which represents a potential mechanism for the formation of lipofuscin granules and CA. Cytoplasmic AGE-modified proteins may be taken up in lysosomes by an autophagic mechanism, but are difficult to degrade because they are protease-resistant. This finding suggests that lipofuscin granules and CA might be formed by similar mechanisms that are different from those involved in the formation of other brain age-related inclusions [48].

We can conclude that there is increase of CA and lipofuscin-bearing neurons in the parahippocampal region of the brain with age, but this increase is not linear. The above cited literature data about the effects of lipofuscin and CA on neuronal metabolism, and the importance of the parahippocampal region in memory function, may support the assumption that the cases of the second group in our study presented some kind of senescence-prone phenotype individuals like the one cited by Shimada et al. [47], and were characterized by at least an altered parahippocampal region metabolism during their lifetime. The presence of the two older groups (second and the third) in our study, in which age was not significantly different but the number of lipofuscin-bearing neurons and CA were, might be explained as the consequence of different brain aging patterns [49]. Finally, such a metabolic state of the parahippocampal gyrus neurons can indirectly support Keller's [4] hypothesis that such senescence-related changes, like CA and lipofuscin deposits, might represent predecessors for more serious changes, like the ones observed in the early phase of AD in the entorhinal area as part of the parahippocampal region [5–15]. Future research should include the development of *in vivo* methods of CA and lipofuscin deposit detection and quantification in the brain of the elderly individuals. Simultaneous application of these methods, and evaluation of such individuals' cognitive status, could lead to a conclusion about the influence of such changes on parahippocampal region functional status and consequent cognitive dysfunction during the aging process.

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