Contents lists available at ScienceDirect

Aging Brain

Monitoring clusterin and fibrillar structures in aging and dementia

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ARTICLE INFO

ABSTRACT

Objective: Clusterin is involved in a variety of physiological processes, including proteostasis. Several clusterin polymorphisms were associated with an increased risk of developing Alzheimer's disease, the world-leading cause of dementia. Herein, the effect of a clusterin polymorphism, aging and dementia in the levels of clusterin in human plasma were analysed in a primary care-based cohort, and the association of this chaperone with fibrillar structures discussed.

Methods: 64 individuals with dementia (CDR≥1) and 64 age- and sex-matched Controls from a Portuguese cohort were genotyped for CLU rs1136000 polymorphism, and the plasma levels of clusterin and fibrils were assessed.

Results: An increased prevalence of the CC genotype was observed for the dementia group, although no significant robustness was achieved. CLU rs11136000 SNP did not significantly change plasma clusterin levels in demented individuals. Instead, clusterin levels decreased with aging and even more in individuals with dementia. Importantly, plasma clusterin levels correlated with the presence of fibrillar structures in Control individuals, but not in those with dementia.

Conclusion: This study reveals a significant decrease in plasma clusterin in demented individuals with aging, which related to altered clusterin-fibrils dynamics. Potentially, plasma clusterin and its association with fibrillar structures can be used to monitor dementia progression along aging.

Introduction

Population aging continues to grow being that around 10% of the world's population is aged 65 or over, a number expected to exceed 16% by 2050 [1,2]. As the population ages several socio-economical and health care challenges arise, including the fact that aging is the major risk factor for developing dementia [3,4].

Dementia encompasses a set of pathological changes, among them loss of memory, language and problem-solving, all are aspects associated with cognitive decline and interfere with daily life activities. Neurodegenerative

disorders, like Alzheimer's disease (AD), Parkinson disease, Lewy bodies or frontotemporal lobar degeneration are among the main causes of dementia in the older population [5]. In fact, late-onset AD (LOAD) is the worldwide leading cause of dementia, being this disease associated with 60 to 80% of all cases [6]. LOAD, accounts for 95% of AD cases, is considered to be essentially sporadic in nature, although the contribution of risk genes such as APOE, the major risk factor identified for LOAD, can be considerable [7,8].

Genome-wide association studies (GWAS) compared millions of single nucleotide polymorphisms (SNP) and identified several additional genetic risk determinants for LOAD [9–11]. Among these is the clusterin (CLU) gene, also known as Apolipoprotein J [12,13]. Secreted clusterin is the most abundant form and can be found in a variety of body fluids, including plasma and cerebrospinal fluid (CSF) [13–15]. In the extracellular space, clusterin serves as a heat shock protein-like chaperone that aids in the folding of secreted proteins [16,17] and is able to form stable complexes with misfolded proteins and peptides to prevent their aggregation or facilitate disposal [18]. Clusterin exhibits a multifunctional nature and is involved in a growing number of pathologies, placing it as a biomarker candidate and promising therapeutic target for different conditions [19]. Of note, clusterin can bind a wide variety of targets, including the amyloid-β peptide (Aβ, the main core of senile plaques in AD) [17,20] or α-synuclein oligomers (the cytotoxic species in several α-synucleinopathies) [21], revealing an important role of this chaperone in extracellular proteostasis and neuroprotection.

Accumulation of misfolded proteins is a common hallmark in many neurodegenerative diseases and clusterin was found to co-localize with different disease-associated extracellular deposits of highly structured protein aggregates, including amyloid plaques [22]. The role of clusterin in AD etiology depends on the interaction with Aβ and tau aggregates. However, its exact contribution to the dynamics of amyloid aggregation and clearance is still ambiguous, with both beneficial and detrimental roles assigned to clusterin [23–26]. Clusterin upregulation was observed in the hippocampus and cortex regions of AD brains [27,28], and elevated clusterin levels can be detected in CSF of affected individuals [29,30]. At a more peripheral level, some groups reported elevated clusterin levels in plasma of AD individuals [31,32]. These levels were associated with disease severity and progression [33], but also with the transition from mild cognitive impairment (MCI) to AD, and with the risk of dementia amongst the elderly [34–36], although inconsistencies have been observed. Several CLU SNP were associated with an altered AD-risk, with the variant rs11136000 being considered the most relevant one, but exhibiting some population-specific differences [37–39]. The first studies addressing CLU rs11136000 SNP, reported that the C allele was associated with an increased risk of developing AD [40], while the T allele was considered protective [41], possibly by genetic variant rs11136000 regulation of CLU expression [42]. Individuals carrying a CC genotype have been associated with increased cognitive decline when compared to TT counterparts [43]. Additionally, genetic risk variants of

rs11136000 have been associated with higher Aβ deposition [44], and with structural brain changes in cognitively normal individuals, occurring years before the appearance of AD symptoms [45,46]. However, it was also reported that the C allele reduces the AD risk [47].

The rs11136000 SNP was also correlated with clusterin protein levels in plasma. Likewise, conflicting data was obtained with distinct authors reporting: (1) an association between lower levels of plasma clusterin and the C risk allele in cognitively healthy individuals [48]; (2) no effect of the rs11136000 SNP in plasma clusterin [33]; (3) or decreased levels in TT homozygotes of the Control group, and increased levels in MCI and AD individuals compared to Controls [31]. Other authors also reported increased plasma clusterin in type-II diabetes mellitus patients with MCI, and that CLU rs11136000 TT genotype is associated with a reduced MCI-risk [49]. Hence, this study addresses the influence of the CLU rs11136000 gene variant in the incidence of dementia in a subpopulation from a primary care-based cohort (pcb-cohort) previously established and characterized by our group [50]. The contribution of this SNP, aging and dementia to the levels of clusterin in plasma samples is likewise assessed. Finally, this study also evaluated the association between clusterin and the presence of fibrillar proteins and peptides in human plasma.

Material and methods

Characterization of the study population

Human blood and plasma samples from the primary health care-based cohort (pcb-cohort), established in the Aveiro region, Portugal [50] were used for this study. The pcb-cohort comprises a total of 568 individuals who fulfilled the inclusion criteria: ≥50 years old, no record of ongoing oncological treatments, absence of psychiatric disorders (excluding depression) or aphasia, and ability to answer the questions during the interview. These individuals were submitted to a battery of cognitive evaluations and dementia screening tests during the interview, regardless of the clinical diagnosis [51]. Herein, 64 samples from individuals screened for cognitive impairment, as determined by Clinical Dementia Rating (CDR) scores ≥ 1, and 64 age- and sex-matched Controls, randomly selected amongst cognitively normal individuals (CDR=0), were analyzed [52]. According to the CDR scale, CDR=0 indicates no dementia, CDR=0.5 suspected, questionable, or very mild dementia, while a CDR≥1 mild (CDR=1), moderate (CDR=2) or severe dementia (CDR=3) [51]. The 64 CDR≥1 individuals were designated as 'dementia' group and include 9 clinically diagnosed Alzheimer's disease (AD) patients, while the 64 cognitively normal individuals were designated as 'Control' group. Characterization of the selected sub-group of individuals is presented in Table 1.

Sample collection

Blood samples were collected in EDTA-tubes, according to standard procedures, and plasma was obtained by centrifugation of blood samples in EDTA gel separator tubes.

Table 1
Characterization of the study group according to age, gender and clinical dementia ratio.

Group	Control		CDR $\geq$ 1	
N	64		64	
Gender	Female	Male	Female	Male
N (%)	42	22	42	22
	(65.6%)	(34.4%)	(65.6%)	(34.4%)
Age (years, mean ± s.d.)	74.9 ± 7.2		75.7 ± 7.8	

Data are presented as N (number of individuals).

Blood and plasma were promptly stored at −80 °C, as previously described [50]. Sample collection was performed in compliance with all guidelines, and approved by the Ethics Committee for Health of the Central Regional Administration of Coimbra (CES of ARS Centro, protocol N°012804-04.04.2012) and by the National Committee for Data Protection (CNPD N°369/2012). All participants gave written informed consent.

CLU genotyping

A 685 bp fragment from CLU rs1113600 polymorphic region was amplified using Phusion Blood Direct PCR Master Mix (ThermoFisher Scientific, USA), according to the manufacturer's instructions. Briefly, 1 μL of blood was mixed with 2x Phusion Blood II DNA Polymerase Master Mix, 1 μL of each primer (Eurogentec, Belgium; Fw: 5́-CCTGGCTTAAAGAATCCACTCATC-3′, Rv: 5′-CAGGGGATTCCTTTGAGATAGAGT-3′, final concentration of 0.1 μM each) and 7 μL of ultrapure DNase distilled H$_2$O. The PCR reaction included a denaturation step at 98 °C for 5 min, followed by 35 cycles of amplification at 94 °C for 1 min, 63 °C for 30sec and 72 °C for 46 sec, and a final extension step at 72 °C for 5 min. The mixture was centrifuged at 1000 g for 3 min and the supernatant transferred into new tubes. Amplification of PCR products was verified by agarose gel electrophoresis.

For CLU rs1113600 SNP analysis, resulting DNA product was precipitated using 1/10 vol of sodium acetate (pH 3 M, pH 5.2) and 2.5 volumes of 100% ethanol. Samples were then centrifuged at 14000 rpm for 20 min, 4 °C, and the pellet washed with 70% ethanol. Following an additional centrifugation, the resulting pellets were dried out and then resuspended in ultrapure DNase distilled H$_2$O. 10 μL of purified PCR products were analyzed by Sanger Sequencing in an expert external laboratory (Stab Vida, Portugal), using the Fw primer 5′-CCTGGCTTAAAGAATCCACTCATC-3′ as described in [53]. Results were analyzed with the BioEdit software (Ibis Biosciences, Carlsbad USA).

Western and slot blot analysis

Clusterin levels in human plasma samples were determined by western blot analysis using an anti-human clusterin antibody (ref. 552886; BD Biosciences). For each sample, 30 μg were prepared in loading buffer and separated by SDS-PAGE. Proteins were blotted into nitrocellulose membranes and incubated with the anti-clusterin primary antibody, overnight at 4 °C, followed by an anti-mouse IgG, HRP-linked, secondary antibody (ref. 7076 s; Cell Signalling), 1 h at R.T. Clusterin was detected with ECL Select (GE Health-

care) in a Chemidoc Touch Imaging System (BioRad Laboratories, Inc.) and the resulting images were analyzed using the Image Lab software (BioRad Laboratories, Inc.). Density values were expressed as arbitrary units. A pool of plasma was used as reference sample in each blot, and across blots, to normalize and compare the obtained results.

The aggregation state of proteins and peptides present in plasma samples from Control and CDR $\geq$ 1 individuals, was evaluated by slot blot analysis using an anti-amyloid fibrils OC antibody (Merck KGaA), as previously described [54]. This conformation-specific and sequence-independent antibody recognizes amyloid fibrils and fibrillar structures but not prefibrillar oligomers or natively folded proteins [55]. Briefly, 10 μg of total plasma protein were resuspended in TBS and transferred to nitrocellulose membranes (GE Healthcare) using a Bio-Dot SF Microfiltration Apparatus (Bio-Rad Laboratories, Inc.). A pool of plasma was loaded in each membrane and used as a reference sample to normalize across results. Ponceau S staining (Sigma-Aldrich) was used as a loading Control. Membranes were incubated with the anti-amyloid fibrils OC antibody, overnight at 4 °C, followed by anti-rabbit IgG, HRP-conjugated, secondary antibody (7074 s, Cell Signaling). Band detection and image acquisition and analysis were performed as described above.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software). Data sets with a normal distribution were analyzed by two-tailed Student's *t*-test and Two-way ANOVA test, and partial correlations analyzed by Pearson *r* coefficient. For data sets that do not follow a normal distribution the non-parametric two-tailed Mann−Whitney *U* test was used, and partial correlations were assessed by Spearman rank correlation. Normality of each data set was tested using the Shapiro-Wilk normality test. Chi-squared test was performed to compare non-continuous variables. Odds ratios (OR) with correspondent 95% confidence intervals (CI) were used to analyze the association of risk alleles or genotypes with dementia. Significance level was set at 0.05 and p-values≤0.05 were considered statistically significant.

Results

Genotypic and allelic characterization of the clusterin rs1113600 polymorphism in the pcb-cohort

Genotypic and allelic characterization of the CLU rs1113600 SNP was carried out in a group of individuals

from the pcb-cohort [50]. The study group included 64 individuals with mild to severe dementia, according to CDR scores and collectively designated as the dementia or CDR≥1, and 64 sex- and aged-matched Controls selected amongst the cognitively normal individuals (CDR=0). Age and gender distribution of the Control and CDR≥1 groups are shown in Table 1. Overall, both groups have the same gender distribution, with an average age of 74.9 ± 7.2 and 75.7 ± 7.8 for Control and CDR≥1, respectively.

Sequencing analysis revealed that from the 64 individuals with mild to severe dementia, a total of 34 were homozygous risk-carriers (CC genotype), 19 were heterozygous risk carriers (CT genotype), and 11 were homozygous non-risk carriers (TT genotype). Comparatively, the Control group comprises 26 homozygous risk-carriers (CC genotype), 25 heterozygous risk-carriers (CT genotype) and 13 homozygous non-risk carriers (TT genotype). Despite the frequency of the CC genotype in the CDR≥1 group (34 out of 64) was higher than in Controls (26 out of 64) (Table 2), no statistical differences were observed in the distribution of CLU rs1113600 genotypes between both groups. Further, increased odds ratios were

Table 2
Distribution of CLU rs11136000 genotype frequencies between the Control and dementia groups.

Genotype	Control N	CDR≥1 N	Total	OR (95% CI)	p-value
CC	26	34	60	1.545 (0.601 – 4.180)	0.469
CT	25	19	44	0.898 (0.313 – 2.291)	0.999
TT	13	11	24	1.000	–
CC	26	34	60	1.656 (0.823 – 3.336)	0.215
TT + CT	38	30	68	1.000	–

Data are presented as N (number of individuals). Genotype frequencies were compared between both groups with Fisher's exact test. OR: odds ratio; CI: confidence interval.
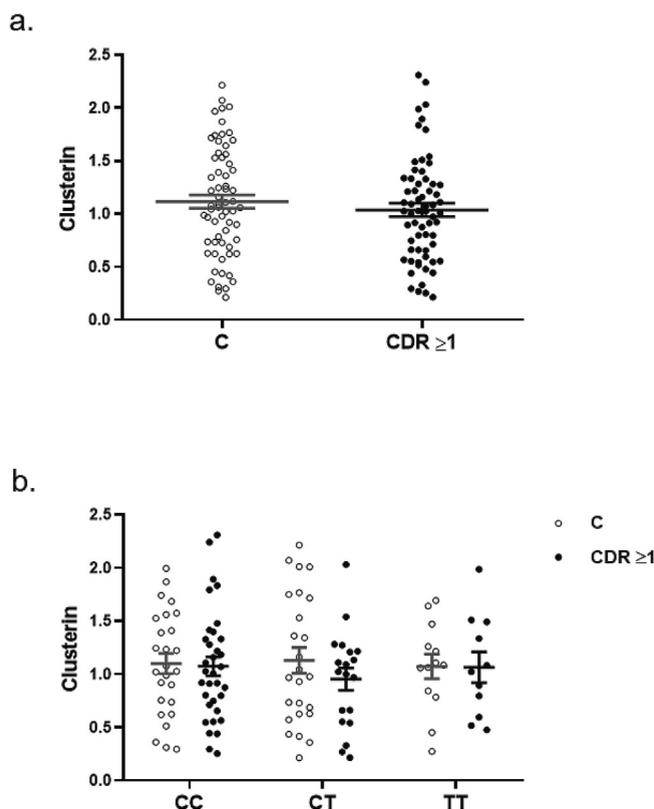
Fig. 1. Levels of Clusterin in plasma of Control and CDR≥1 individuals. The levels of clusterin were evaluated in plasma of Control (○) and CDR≥1 individuals (●) as whole groups (**a**), and according to the CLU rs1113600 SNP genotype (**b**). A t-test was applied to compare the Control and CDR≥1 groups (p = 0.3515), while a Two-Way ANOVA with multiple comparisons was used to test the effect of the genotypes in the two groups (p=0.7199). All samples were normalized to the reference sample and clusterin levels are represented as fold changes. Bars represent mean ± s.e.m.
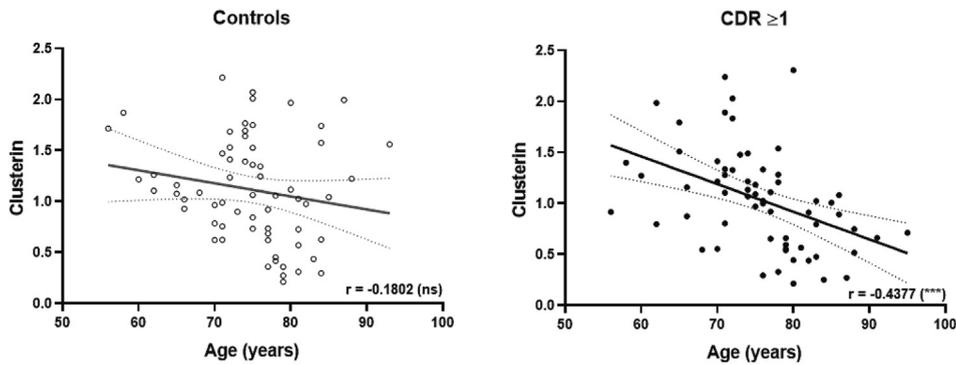
Fig. 2. Plasma levels of Clusterin in Control and CDR≥1 individuals, with aging. Levels of plasma clusterin were assessed by immunoblotting and plotted according to the age of each individual. Pearson's coefficient (Pearson r) was applied to test the correlation of both groups, (a) Controls (○) and (b) CDR≥1 (●), with aging. In Controls, (ns) stands for non-significant and p=0.1543; in CDR≥ 1, p=0.0003 (***). All samples were normalized to the reference sample and clusterin levels are represented as fold changes. Lines represent a linear regression fit with corresponding 95% confidence bands (dashed lines).

observed for the CC genotype when compared to TT genotype (OR=1.545, p=0.469), and for the recessive model CC vs TT + CT (OR=1.656, p=0.215), although none has reached statistical significance (Table 2).

Clusterin plasma levels in Control and demented individuals

Clusterin levels in the plasma of CDR≥1 individuals and the corresponding age- and sex-matched Controls were assessed by western blot. No significant differences (p=0.3515) were observed in the plasma levels of clusterin between both groups (Fig. 1a), although a slightly lower tendency could be detected in the CDR≥1 group. The impact of CLU rs11136000 SNP in clusterin plasma levels was also evaluated in the pcb-cohort, by addressing the clusterin levels in both groups according to genotype (Fig. 1b). Data suggests that the presence of the 'C' allele, a potential risk factor in AD, either in homozygous 'CC' or heterozygous 'CT' individuals is not associated to fluctuations in the plasma levels of this protein. The average levels of plasma clusterin are identical independently of the genotype (p=0.9059). Altogether, the presented data indicates that CLU rs11136000 SNP genotype does not affect the circulating levels of human plasma clusterin.

Plasma clusterin levels in aging and dementia

To address if aging can affect plasma clusterin levels, the latter was monitored and analyzed in an age-dependent manner, for individuals in both study groups. Data presented revealed a week negative correlation (Pearson's r=−0.1802, p=0.1543) between clusterin plasma levels and aging in Control individuals (Control group, Fig. 2). A stronger decline in plasma clusterin levels was observed in samples from individuals with mild to severe dementia. Analysis of the CDR≥1 group revealed a significant negative correlation between clusterin levels and age (Pearson's r=-0.4377, p=0.0003), which might be related to dementia progression (Fig. 2).

*Clusterin correlation with fibrillar structures in human plasma*

Clusterin assists in the folding of secreted proteins and functions as an extracellular chaperone to prevent the aggregation of non-native proteins, including in blood. A previous study by our group evaluated plasma protein aggregation in non-demented individuals (CDR=0) with aging [54]. A similar approach was here employed to explore how dementia or clusterin change the aggregation status of plasma proteins. Using a conformation-specific and sequence-independent antibody directed against fibrillar structures (OC), the levels of amyloid fibrils and fibrillar oligomers were evaluated in the plasma of both groups. In general, no significant differences were observed for these aggregated species after immunodetection with the OC antibody (p = 0.7190), despite the fact that even though the average level of fibrillar structures in CDR≥1 samples is slightly higher than in Controls (Fig. 3).

Since clusterin is able to prevent protein aggregation and is involved in the clearance of extracellular misfolded proteins, correlation analyses between the plasma levels of clusterin and fibrils were carried out. In the Control group, a significant moderate negative correlation was observed (Spearman r=−0.4017, p=0.0010), indicating that a higher abundance of clusterin is associated with fewer fibrillar species (Fig. 4). Of note, this correlation was lost in the CDR≥1 study group (Spearman r=−0.1250, p=0.3252), and was no longer significant (Fig. 4).

Discussion

The relationship between clusterin polymorphisms and dementia is controversial, with authors either reporting an association with increased or reduced risk of dementia. To our knowledge, this is the first study addressing the association between CLU rs1113600 SNP and cognitive impairment in a Portuguese sub-population. Cognitive impairment may represent a transition stage to AD, since annualy 10–15% of MCI cases can progress to AD [56], or other dementia-associated pathologies.

Genotyping of the pcb-cohort sub-groups did not allow to determine the exact contribution of the 'C' allele to the
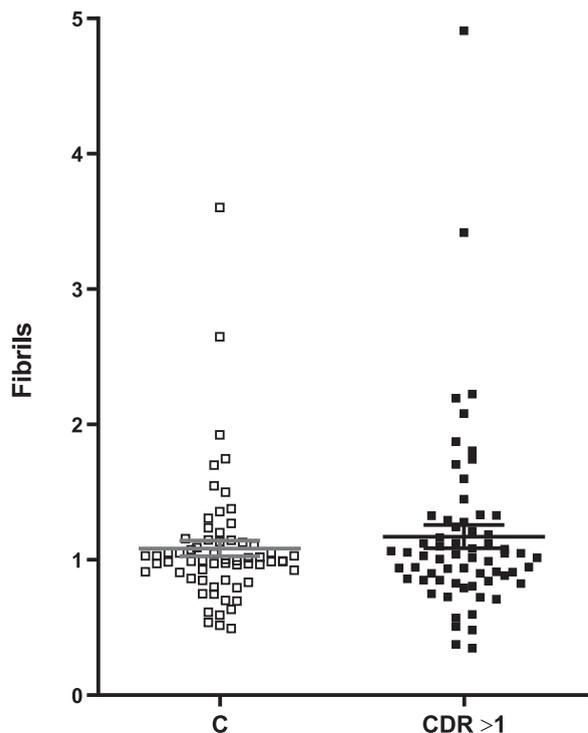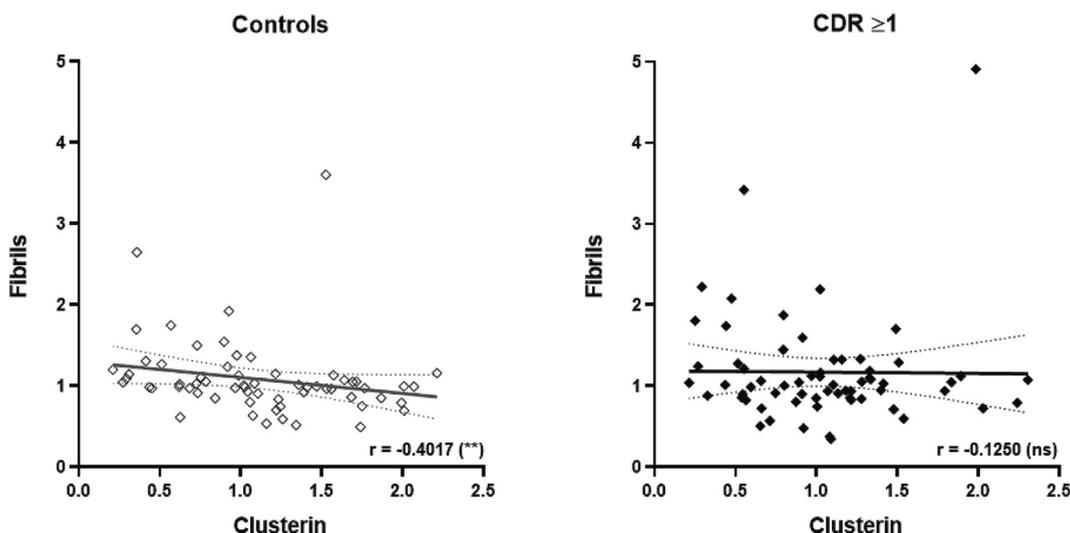
Fig. 3. Levels of Fibrillar structures in Control and CDR≥1 individuals. The levels of fibrils were evaluated in plasma of Control (□) and CDR≥1 individuals (■) using a two-tailed Mann-Whitney test (p=0.7190). All samples were normalized to the reference sample and fibrils levels are represented as fold changes. Bars represent mean ± s.e.m.

development of dementia in this Portuguese population. Although it was observed a higher prevalence of CC risk homozygotes in the dementia group; and higher OR analy-

sis for CC genotype when comparing to the TT genotype, no significant robustness was achieved to support the association between CC genotype and dementia in this study. An increased risk for dementia was originally attributed to the C allele [40], which flagged CLU rs11136000 SNP in GWAS for risk polymorphisms associated to AD [38,39]. Consistently, other authors also reported an association of the TT genotype with decreased risk of MCI [49], while the C risk allele may be associated with faster memory decline in the transition between MCI and AD [43]. Nevertheless, opposite data has been recently reported, suggesting that the C allele in CLU rs11136000 SNP could actually play a role in reducing the risk of AD [47]. It should be noted, however, that the association of this SNP with AD exhibits population-specific variability, being less consistent in Asian cohorts [57], than it is for European cohorts [37]. Classifying a SNP as protective or risk factor is not simple, and indeed, different observations could be associated with the presence of other SNPs at distinct loci.

No correlations were found between the rs11136000 genotypes and the plasma clusterin levels of the Control and dementia groups, in agreement with observations from other groups [33,49]. Nonetheless, at this level, contradictory data has also been reported. While some studies showed an increase in plasma clusterin in TT homozygotes of Control individuals [48] others revealed an opposite effect [31]. None of these works detected genotype-associated differences in plasma clusterin of AD individuals.

Further, while some authors reported increased plasma clusterin levels in AD and even MCI individuals, compared to cognitively normal Controls [35,58], others also found identical levels of this protein in the plasma of AD and Control individuals. In agreement with the later, the results reported herein reveal no differences in plasma clusterin levels between Controls and the dementia group. A recent

Fig. 4. Correlation between clusterin and fibrillar structures. The levels of clusterin and fibrils were independently evaluated in plasma of (a) Control (◇) and (b) CDR≥1 individuals (◆), and matched values plotted. Correlations were evaluated by Spearman coefficients (r), with (**) p=0.0010 for Controls and (ns) p=0.3252 for CDR≥1. All samples were normalized to the reference sample and fibrils vs clusterin levels are represented as fold changes. Lines represent a linear regression fit with corresponding 95% confidence bands (dashed lines).

meta-analysis analyzing 7228 individuals, including 1936 AD cases, also found no association between plasma clusterin levels and the diagnosis, risk, or severity of AD [59].

Since aging is the major risk-factor in several dementia-associated pathologies, it was also analyzed its impact in plasma clusterin. Results demonstrate a negative association between plasma clusterin and the age of individuals, as reported by others [36]. The data herein presented provides evidence for an age- and dementia-associated decrease in plasma clusterin levels, which was more pronounced in dementia group than in cognitively normal individuals. The differences observed between the Control and dementia groups support the hypothesis of clusterin as an important player in the human body's strategy to cope with pathological processes underlying the onset and progression of dementia.

The protective role of clusterin in dementia and other proteinopathies seems to depend on its ability to bind misfolded proteins and prevent aggregation, thus maintaining extracellular proteostasis. Previous work by our group demonstrated that the plasma proteome undergoes conformational changes with aging, in cognitively normal individuals. Particularly, an age-related increase in fibrillar structures was detected [54]. Herein, no significant differences were found in the plasma levels of fibrillar proteins and peptides between Control and individuals with dementia. One cannot exclude that during disease progression the clearance rate of protein aggregates in the brain may change, but it is difficult to monitor such differences. Consistent with this observation, Chen and colleagues reported that plasma Aβ42 increases in the MCI phase and decreases just before clinical AD onset [60].

The association between clusterin and fibrillar species in human plasma was also evaluated, and a significant inverse correlation was detected in the Control group. This is consistent with the clusterin chaperone activity to preserve extracellular proteostasis [16]. Physiological concentrations of clusterin were reported to efficiently prevent the precipitation of proteins in human serum [61], supporting the association between higher levels of this protein and decreased fibrils. Remarkably, the association between clusterin and fibrils is disrupted in the dementia group, suggesting an impaired ability to properly maintain proteostasis in individuals with dementia. It cannot be excluded that the nature of the aggregates produced in demented individuals could also be slightly different (e.g., richer in fibrillar structures), and therefore more difficult to clear via clusterin. Hence, these analyses sustain the idea that pathological changes underlying dementia can disturb the proteostasis equilibrium in human plasma. A similar effect has been reported in patients with controlled hypertension [62]. Such phenomenon could be associated with changes in the plasma proteome of individuals with dementia, including differences in the aggregation state of misfolded proteins and/or impaired capacity of clusterin to prevent the formation or promote clearance of such aggregates.

This work supports that plasma clusterin levels are not affected by the different variants of the AD risk-associated CLU rs1113600 SNP but change with aging, exhibiting a more evident decline in individuals with cognitive impairment. Noticeably, clusterin levels significantly correlated with the amount of plasma fibrils in Controls, a correlation lost in dementia individuals possible reflecting a decrease in this chaperone activity. Further validation in distinct cohorts and larger populations, as well as the longitudinal assessment of plasma clusterin levels and its correlation with fibrillar structures, could be valuable for monitoring dementia. In combination with other diagnostic approaches, this can potentially contribute to evaluating the risk of developing dementia and its progression with aging.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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