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HLA-A23/HLA-A24 serotypes and dementia interaction in the elderly: Association with increased soluble HLA class I molecules in plasma

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MHC class I molecules regulate brain development and plasticity in mice and HLA class I molecules are associated with brain disorders in humans. We investigated the relationship between plasma-derived soluble human HLA class I molecules (sHLA class I), HLA class I serotypes and dementia. A cohort of HLA class I serotyped elderly subjects with no dementia/pre-dementia (NpD, $n = 28$), or with dementia (D, $n = 28$) was studied. Multivariate analysis was used to examine the influence of dementia and HLA class I serotype on sHLA class I levels, and to compare sHLA class I within four groups according to the presence or absence of HLA-A23/A24 and dementia. HLA-A23/A24 and dementia, but not age, significantly influenced the level of sHLA class I. Importantly, the concurrent presence of HLA-A23/A24 and dementia was associated with higher levels of sHLA class I ($p < 0.001$). This study has shown that the simultaneous presence of HLA-A23/HLA-A24 and dementia is associated with high levels of serum sHLA class I molecules. Thus, sHLA class I could be considered a biomarker of neurodegeneration in certain HLA class I carriers.

KEYWORDS
Alzheimer's, biomarkers, dementia, immunoregulation, MHC, neurodegeneration, Parkinson's, soluble HLA class I

1 | INTRODUCTION

Besides being central players in the immune response of CD8+ T cells to self and non-self-antigens, human major histocompatibility class I molecules (MHC class I, HLA class I in humans, H-2 class I in mice) are crucial

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modulators of brain organogenesis and risk factors for the development of neurological disorders.[1–3] Based on polymorphism and tissue expression, HLA class I molecules can be classified into classical (HLA-A, B, C) and non-classical (HLA-E, F, G).[4] Mature classical HLA class I molecules are expressed at the cell surface of every nucleated cell and can exist in a physiological equilibrium between two main forms. During normal quiescent conditions, they exist as trimers of a heavy chain, which extracellular part is structured into three domains (α1, α2, and α3), a light chain (β2m) and a small peptide, known as closed conformers.[5] During physiological conditions associated with cell activation, increased metabolic activity, stress, or inflammation, a pool of cell surface HLA class I molecules can exist as β2m-free/peptide-empty heavy chains, known as open conformers.[5] Both cell surface conformations may be shed into the extracellular milieu and circulate as soluble HLA class I molecules (sHLA class I).[6–8] The formation of HLA class I open conformers is paralleled by the release of β2m, which can also be found in many biological fluids, including the cerebrospinal fluid, and has been associated with impaired cognitive function, Alzheimer's disease and dementia.[9]

Studies using mouse models where the expression of MHC class I molecules was genetically manipulated, have conclusively shown deviations in fundamental biological processes of the central nervous system (CNS), including brain synaptic plasticity, neurogenesis, maintenance of dendritic complexity, and hippocampal synapse density.[1–3,10,11] Although similar studies in humans are unfeasible, case–control studies with patients harboring different CNS-related pathologies have shown associations with discrete HLA class I alleles. Thus, HLA-A2-carriers had an earlier onset of Alzheimer's disease (AD), while carriers of HLA-A1 presented a delayed AD development.[12,13] In a recent study using next-generation sequencing and estimation of the binding affinity of self-antigens, HLA-A2 and self-antigen load were associated with the age of AD onset.[14] Modulation of AD by HLA-A2 alleles appears to be mediated by the induction of differences in hippocampal volume.[15] Also, the risk of Parkinson's disease (PD) has been positively associated with the *B*07_C*07* haplotype.[16] Overall, these studies suggest that in humans the HLA class I molecules are factors that must be considered when performing studies of brain function and homeostasis.

Despite the body of knowledge from animal and human studies, the exact role played by MHC class I molecules in modulating biological processes of the CNS and their involvement in neurological disorders is a matter of intensive investigations.[17] Importantly, in the mice studies both cell surface and soluble forms of H-2 class I molecules were implicated in synaptic plasticity and neurogenesis, likely through *cis-trans*-interactions with surface receptors important for brain function.[18–20] In this respect, studies characterizing the levels of soluble HLA class I have primarily focused on the non-classical molecules HLA-G and HLA-E in autoimmunity, cancer, infection, diabetes, and transplantation.[21,22] Surprisingly, studies characterizing the levels of classical sHLA class I molecules in the context of deficient cognitive function, Alzheimer's and Parkinson's diseases, and dementia in humans are lacking. Hence, we aimed to explore a possible association between the levels of classical sHLA class I molecules and the presence or absence of dementia in a cohort of HLA class I serotyped elders.

2 | METHODS

2.1 | Ethical approval

All study participants, or their legal representatives, provided informed consent, and the study protocol was approved by the Institutional Review Board (IRB) in accordance with the Declaration of Helsinki (Ref. Number CE-UBI-Pj-2017-012). All obtained data from elderly subjects (personal, clinical, and evaluation data) were stored according to data protection regulations and legal directives.

2.2 | Subjects and criteria classification

Fifty-six elderly people from a total of 86 elderly subjects from retirement homes and daycare centers of the Beira Interior region of Portugal were studied. Elderly subjects were evaluated by a trained team that assessed them using the Global Deterioration Scale (GDS),[23] a revised Addenbrooke's Cognitive Examination (ACE-R),[24] and clinical data. ACE-R can be applied in a short time (15–20 min) and, in this scope, constitutes a useful tool for the detection of dementia syndromes. Good levels of sensitivity have been reported in the distinction between healthy controls and patients with some type of dementia in initial stages.[25–27] Exclusion criteria were subjects that withdrew from the study, infection apart from cytomegalovirus (CMV), diagnosis of previous stroke, neoplasms, autoimmune diseases, psychiatric disorders, epilepsy, trauma, or absence of clinical data. CMV seropositivity was included because its prevalence is high among elderly people and several studies have associated CMV infection with the risk of moderate-to-severe dementia.[28]

The elderly subjects under study were initially classified into three discrete groups, as previously described.[29] The first group included the elderly without cognitive

impairment (NCI, $n = 13$, GDS stages 1/2, ACE-R values indicative of the absence of cognitive impairment, that is, 106.49 ± 3.05% of the minimal normal score, and without clinical evidence of disease with cognitive impairment). The second group included elders with mild cognitive impairment (MCI, $n = 15$, GDS stage 3, and ACE-R values slightly lower than the minimal considered as normal, that is, 73.6 ± 3.61% of the minimal normal cognitive impairment level). The third group included the elderly with moderate-to-severe cognitive impairment plus six additional elderly subjects not included in the previous study (CI, $n = 28$, GDS stage 4 or above). This group includes 19 elderly subjects that performed ACE-R (46.67 ± 4.12 of the minimal normal cognitive impairment level), including six elderly subjects with Parkinson's disease (PD) diagnosed by a generalist physician, and 9 elderly subjects with clinical information indicating the existence of Alzheimer's disease (AD) or dementia, for which there was not possible to apply the ACE-R. Afterward, a cluster analysis with the Two-Step method[30] was performed resulting in the creation of two clusters with fair quality. The first cluster included NCI and MCI subjects and the second cluster included all the CI patients. Following Reisberg criteria,[31] the first cluster was named the Normal or pre-Dementia group (NpD), while the second cluster was named the Dementia group (D).

2.3 | Blood sample collection

Blood samples from each volunteer were collected in EDTA tubes and assigned a double identification code, according to the IRB-approved proposal. Coded samples were processed within 2–3 h of collection to obtain peripheral blood mononuclear cells (PBMC) and plasma. Plasma samples were obtained after centrifugation of 10 mL of fresh blood collected in EDTA tubes at 3000 rpm for 15 min, at room temperature. The collected plasma was centrifuged again at 6000 rpm for 5 min at room temperature and 1 mL aliquots were cryopreserved at −80°C for later studies. Prior to sHLA class I quantification, plasma samples were thawed and centrifuged at 6000 rpm for 5 min at room temperature to remove contaminating platelets or other components in order to prevent interferences with assay performance. All subjects under study were characterized for HLA class I serotype and CMV seropositivity, and described as follows.

2.4 | HLA determination

All subjects under study were characterized for HLA class I serotype as previously described.[29] Briefly, DNA was extracted from peripheral blood using the MagAttract® DNA Blood Midi M48 kit or QIAamp DNA Stool Mini Kit (QS). HLA typing was accomplished using One Lambda® LABTypeSSO kits at low resolution level (serology equivalent) for HLA-A, -B, -C, -DRB1, -DQA1, and -DQB1 loci followed by Luminex® xMAP® technology. Data were deduced in Fusion v4.2 software and are presented at serological equivalent or at antigen allele level when there is no serological equivalent. The frequencies of the HLA-A, HLA-B and HLA-C serotypes in the 56 elders are summarized in Supplemental Table 1A.

2.5 | Cytomegalovirus seropositivity

All subjects under study were characterized for Cytomegalovirus (CMV) seropositivity as previously described.[29] Briefly, cryopreserved plasma samples were thawed and anti-CMV IgG antibodies detected by using 96-well microplate ELISA kits (Demeditec), according to manufacturer instructions. Tests were performed in duplicate, and the amount of CMV-specific IgG antibody bound calculated using a BioRad xMark™ Microplate Absorbance Spectrophotometer. The concentration of IgG antibodies was calculated by comparing to a reference curve obtained with calibrators (i.e., human serum diluted with PBS, with 1, 10, 30, and 90 U/mL of anti-CMV IgG antibodies) following manufacturer instructions.

2.6 | Quantification of sHLA class I

Total soluble HLA (sHLA) class I molecule levels in plasma samples were determined using a solid phase sandwich enzyme-linked immunosorbent assay—ELISA (Pure Protein, LLC, OK). In brief, ELISA plates (Nunc 439454, ThermoFisher Scientific) were coated overnight at 4°C with anti-human HLA class I monoclonal antibody W6/32 at 12.5 μg/mL in phosphate coating buffer (10 mM NaH_2PO_4, 10 mM Na_4HPO_4, pH 7.2). Plates were washed five times using Phosphate Buffered Saline and Tween-20 (PBS-T; 1xPBS: 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 NaCl, 2.68 mM KCl, pH 7.4, 0.05% Tween-20) as wash buffer and then blocked with 3% Bovine Serum Albumin (BSA, in 1× PBS) for 2 h at room temperature. After washing, plates were incubated for 1 h at room temperature with either 50 μL of plasma sample or standard *sHLA-A*02:01* positive control, all diluted 2–256-fold in 3% casein (BioPLUS, bioWORLD, OH). After a five-time wash, a biotin-conjugated rabbit polyclonal antibody recognizing the β2m subunit of captured sHLA (1.25 μg/mL) was added for another hour. Next, an HRP enzyme (VECTASTAIN Elite ABC-HRP Kit, PK-6100,

TABLE 1 Relevant demographic and clinical data of the elderly volunteers studied.

Variable	NCI ($n = 13$)	MCI ($n = 15$)	CI ($n = 28$)	p-value
Age (years old), Mean ± SEM	83.7 ± 2.4	82.5 ± 2.1	83.6 ± 1.7	0.906[a]
Sex (Male/Female), n	8/5	3/12	6/22	**0.029**[b]
CMV seropositivity (n, %)	12 (92.3)	15 (100.0)	28 (100.0)	0.232[b]
sHLA class I (ng/mL), Mean ± SEM	343.3 ± 32.1	317.5 ± 25.2	456.8 ± 41.8	0.081[c]
HLA-A23 or HLA-A24 (n, %)	3 (23.1)	2 (13.3)	11 (39.2)	0.138[b]

Note: Bold values, mean statistically significant.

Abbreviations: CI, moderate-to-severe cognitive impairment; MCI, mild cognitive impairment; NCI, non-cognitive impairment.

[a]One-way ANOVA test.

[b]Fisher-Freeman-Halton Exact test.

[c]Kruskal-Wallis one way-ANOVA. n, number of subjects; SEM, Standard Error of the Mean.

Vector Laboratories, CA) was transferred to the wells according to the manufacturer's instruction with a previous wash step. After a final wash, a signal was generated by adding an OPD substrate (ThermoFisher Scientific, MA) for 15 min, stopped by adding 3N H_2SO_4. The plate was read at 490 nm using a spectrophotometer (BioRad, CA). Data analysis was performed using GraphPad Prism 8.0 Software (GraphPad Software, CA) by applying four Parameter Logistics (4PL) to compute dose–response curves. Sera sHLA concentrations were deduced from obtained half maximal effective concentration (EC_{50}) values from the samples compared to the standard.

2.7 | Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics software (version 28) and statistical significance was defined as $p < 0.05$. Graphs were done using GraphPad Prism 8 software (GraphPad Software, San Diego, CA) and IBM SPSS. Continuous variables were expressed as the mean ± SEM (standard error of the mean). The continuous variables were compared between cognitive status groups (NCI, MCI, CI) using one-way analysis of variance (ANOVA) for variables with a normal distribution (i.e., age), and the Kruskal-Wallis one-way ANOVA for variables with a non-normal distribution (i.e., sHLA class I). Fisher-Freeman-Halton Exact test was used to compare column proportions in the frequencies of categorical variables (i.e., HLA class I serotypes, sex, and CMV seropositivity) of unrelated samples. Fisher Exact test was used to compare columns proportions in the frequencies of HLA class I serotypes and Dementia status (2 × 2 contingency tables). In each comparison, the odds ratio (OR) along with the 95% confidence interval (95% CI) was also calculated, having the NCI or NpD groups as reference categories when three (NCI, MCI, and CI) or two (NpD and D) groups were compared, respectively (see Suppl. Table 1). Pearson's or Spearman's ρ correlations were used to examine correlations between variables with normal (age, log-transformed sHLA class I) or non-normal distribution (sHLA class I), respectively. Identification of subgroups within our cohort was done by using the Two-Step cluster analysis,[30] using two categorical variables (Cognitive status and HLA class I serotype) and two standardized scores of continuous variables (Age and sHLA class I). The two new groups/clusters generated a new categorical variable, designated Dementia status (Normal/Pre-Dementia vs. Dementia). Mann–Whitney U Test was used to compare differences in sHLA class I between two independent groups (i.e., HLA class I serotypes, Dementia Status, or sex). Two-way Analysis of Covariance (ANCOVA) was used to examine the influence of age, dementia status, and HLA class I serotype on the levels of sHLA class I or log-transformed sHLA class I in plasma samples. However, only sHLA class I data are presented in the Results section because although there were deviations from normality in the analysis of the sHLA class I data, due to the existence of outliers, the same conclusions were obtained using ANCOVA with log-transformed sHLA class I data. The level of sHLA class I was also compared between four groups defined according to the presence of HLA-A23 or HLA-A24 serotypes and Dementia (No/No, No/Yes, Yes/No, Yes/Yes) using ANOVA with the Gabriel Post Hoc test multiple comparisons.

3 | RESULTS

Table 1 shows selected demographic, biochemical, and genotypic data from the subjects under study. Based on the accumulated evidence, a higher frequency of females versus males were observed in the moderate-to-severe cognitive impairment group (CI). Other findings are worth pointing out. First, neither age nor CMV seropositivity differed between groups (Table 1). Second, a trend for increased values of sHLA class I was observed in the CI group (Figure 1 shows illustrative sHLA class I

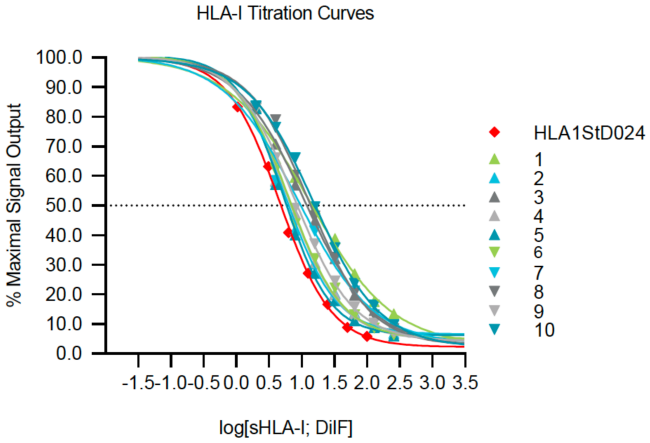

FIGURE 1 Illustrative titration curves for the standard and 10 samples. The graph shows a dose–response experiment with sigmoid functions for the HLA-A standard (red diamonds, HLA1StD024), and 10 samples. The x-axis represents the different dilutions in a log-scale, and the y-axis shows the signal response as the % of the maximal signal output. The $LogEC_{50}$ for the standard serving as reference is set to 0.000 and the EC_{50} factor equals 1.000 (10^0), which is corresponding to the sHLA class I concentration retrieved by the sigmoid curve (in this case 217.0 ng/mL). The $logEC_{50}$ shift is retrieved for each sample, and the EC_{50} shift factor calculated, and the corresponding concentrations are determined. For example, sample number 10 has a $logEC_{50}$ shift equal to 0.5214 [$LogEC_{50}$ (sample)—$LogEC_{50}$ (reference)], which corresponds to a EC_{50} shift Factor of 10^0.5214 = 3.322, and thus a concentration is calculated: 3.322 × 217.0 ng/mL = 720.8 ng/mL. EC_{50}, half maximal Effective Concentration.

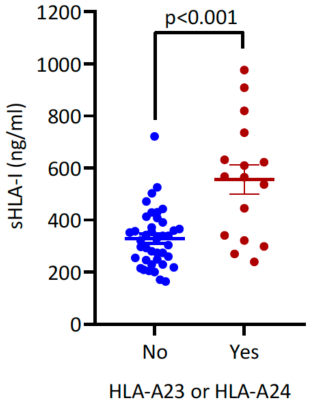

FIGURE 2 Effect of the HLA-A23/HLA-A24 serotypes on the levels of sHLA class I. Soluble levels of HLA class I in the plasma of elderly subjects grouped according to the absence (No) or presence (Yes) of the HLA-A23 or HLA-A24 serotypes, showing statistically significantly higher levels in elders carrying the HLA-A23 or HLA-A24 serotypes. Horizontal and vertical bars represent the mean and standard error of the mean (SEM), respectively. Mann-Whitney U Test. ANOVA, Gabriel Post Hoc test for multiple comparisons; *$p < 0.001$.

titration curves). Third, the high frequency of subjects that are HLA-A23 or HLA-A24 seropositive in the CI group ($n = 11$) when compared to the NCI ($n = 3$) and MCI ($n = 2$) groups. Analysis of the frequencies of the different HLA class I serotypes among the elderly groups showed a significant increase in the HLA-A32 serotype in the NCI group when compared to the CI group (Suppl. Table 1A). Analysis of the odd ratios between the three groups revealed that HLA-A32 appears to confer protection from cognitive impairment (Suppl. Table 1A). In contrast, the frequencies of the HLA-B38 and HLA-C12 serotypes were increased in the MCI group when compared to the CI group (Suppl. Table 1A). The higher frequency of the HLA-C12 serotype was also observed in NpD comparing with the D group (Suppl. Table 1B). In line with these results, odds ratio analysis between NpD and D groups showed that the HLA-C12 serotype also appears to confer protection from dementia (Suppl. Table 1B). Despite these associations, we could not find any influence of these HLA class I serotypes on the level of sHLA class I (data not shown).

In clear contrast, even though the frequency of the HLA-A23 and HLA-24 serotypes was not significantly different between the study groups, elderly subjects with the HLA-A23 or HLA-A24 serotypes had significantly higher levels of sHLA class I molecules than subjects without these serotypes (555.2 ± 56.9 ng/mL vs. 328.3 ± 17.5 ng/mL, $p < 0.001$, Mann–Whitney U Test, Figure 2). Although the CI group was significantly enriched in females (Table 1, $p = 0.029$), the levels of sHLA class I were not different between males and females (390.7 ± 55.4 ng/mL vs. 394.2 ± 26.1 ng/mL, $p = 0.417$, Mann–Whitney Test). Interestingly, the HLA-A23 and HLA-A24 serotypes contain the Bw4 epitope, an amino acid sequence in the α1 domain responsible for the interaction with the inhibitory KIR3DL1 receptor that is also present in other HLA-A and HLA-B serotypes.[32,33] Analysis of the frequencies of the Bw4 epitope within the different elder groups revealed an equal distribution (Suppl. Table 2).

In view of the results obtained, we next wanted to ascertain for the existence of clusters within our cohort. In order to do that, we applied the Two-Step analysis to the categorical variables: cognitive status (NCI, MCI, and CI groups) and HLA class I serotype (with or without HLA-A23/HLA-A24), and to the continuous variables: sHLA class I and age. This analysis produced two cohesive and well-separated clusters (Suppl. Figure 1). The first cluster included the elderly subjects without cognitive decline or with mild cognitive decline, whereas the second cluster included the elderly subjects with moderate to severe cognitive decline. After applying the criteria developed by Reisberg,[31] which provide an overview of

TABLE 2 Interaction between HLA serotypes and dementia[a].

	Main Effect			Interaction
Variable	**Age**	**HLA-A23 or HLA-A24**	**Dementia**	**HLA-A23 or HLA-A24 and dementia**
sHLA class I	$p = 0.569$	$p < 0.001$	$p = 0.001$	$p = 0.003$

[a]Two-way ANCOVA controlling for age, with HLA class I serotype and dementia as independent variables. Adjusted R^2 0.442.

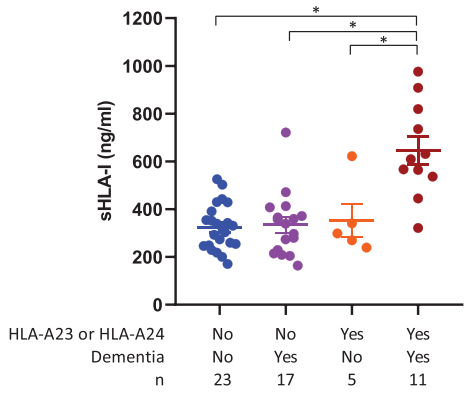

FIGURE 3 Effect of HLA-A23 or HLA-A24 serotypes and dementia on the levels of sHLA class I. Soluble levels of HLA class I in the plasma of elderly subjects grouped according to the cognitive status and HLA-A23 or HLA-A24 serotypes. Statistically significant differences were observed between elders having A23/A24 serotypes and dementia (YES/YES group), and each one of the other groups, that is, elders having the A23/A24 serotypes but not dementia (YES/NO group, $p < 0.001$), elders not having the A23/A24 serotypes but having dementia (NO/YES group, $p < 0.001$), and elders not having neither the A23/A24 serotypes nor dementia (NO/NO group, $p < 0.001$). Horizontal and vertical bars represent the mean and standard error of the mean (SEM), respectively. ANOVA, Gabriel Post Hoc test for multiple comparisons; (*$p < 0.001$; n, number of subjects in each group).

the stages of cognitive function for those suffering from a primary degenerative dementia, we designated the first cluster as the Normal/Pre-Dementia group (N/pre-D, $n = 28$), and the second as the Dementia group (D, $n = 28$). Bivariate analysis revealed that the Dementia group exhibited higher levels of sHLA class I when compared to the Normal/Pre-Dementia group (456.8 $\pm$ 41.8 ng/mL vs. 329.5 $\pm$ 19.9 ng/mL, $p = 0.028$, Mann–Whitney Test). Further, we analyzed the effect of HLA class I serotype, Dementia, and the interaction between these variables, on the level of sHLA class I in plasma samples of the cohort, by using the ANCOVA model. Although we did not find a correlation between age and sHLA class I ($\rho = 0.163$, $p = 0.229$), this attribute was included as a covariate since it has been reported to be associated with increased levels of sHLA class I. The analysis unveiled a major interaction between HLA-A23/HLA-A24 and Dementia with a significant impact on the level of sHLA class I molecules ($p = 0.003$, Adjusted R^2 0.442; see Table 2). Additional analysis revealed that subjects with both HLA-A23/HLA-A24 serotypes and Dementia presented the highest sHLA class I concentrations in their plasma samples (Figure 3, $p < 0.001$), suggesting that the simultaneous presence of HLA-A23/HLA-A24 and Dementia, but not each component separately, contribute to the high levels of plasma sHLA class I in the cohort of elderly subjects studied.

4 | DISCUSSION

By applying an enhanced in-house sandwich ELISA process capable of measuring sHLA cIass I levels for a wide range of concentrations, we demonstrate that elderly people with an HLA-A23 or HLA-A24 serotype in combination with dementia have higher sHLA class I levels in plasma than age-matched elders with only one of the categorical variables. To the best of our knowledge, this is the first time evidence is being presented of an interaction between specific classical HLA class I alleles and dementia that shows an impact on the amount of sHLA class I present in the plasma of elderly people. In our analysis, we included age as a covariate because it has been previously reported to be associated with increased levels of sHLA class I molecules.[34] However, we were unable to show any correlation between sHLA class I and age in our cohort, possibly due to the uneven distribution toward higher aged subjects. In addition, a statistically significantly higher frequency of females versus males was observed in the moderate-to-severe cognitive impairment group. Such findings do not come unexpected and can be explained on the accumulated evidence demonstrating differences between women and men in brain disorders.[35] Moreover, demographic data of our elderly subjects from Portugal show a higher survival rate for females over males at all age ranges. This observation is even more pronounced in elderly subjects above 85 years old, thus emphasizing a higher female prevalence within our cohort (see Suppl. Figure 2) and suggesting that the higher frequency of women within the CI group is likely due to the longer life expectancy of women rather than

sex-specific risk factors for the disease. On the other hand, we found that certain HLA class I serotypes, namely HLA-A32 and HLA-C12 were more prevalent among elderly without moderate-to-severe cognitive impairment, suggesting that they may confer protection against neurodegeneration.

In summary, the results of this study strongly suggest that the presence of specific HLA class I *loci* proteins plus a trigger (i.e., substantial neurodegeneration leading to dementia), but not each factor individually, are required to see an increase in the levels of sHLA class I in plasma. The ELISA assay is built on the preference to recognize mature complexed and structurally intact HLA class I from the classical HLA-A, HLA-B, and HLA-C alleles. Though the ELISA kit used may also recognize non-classical sHLA class I molecules (i.e., sHLA-G, sHLA-E and sHLA-F) numerous scientific reports have demonstrated that plasma/serum levels of soluble HLA-G, HLA-E, and HLA-F molecules are one to several orders of magnitude lower than the levels of classical sHLA-A, B and C molecules (ranging between ~1.0 and 32.0 ng/mL),[22] strongly suggesting that our results for sHLA class I molecules (ranging between 164.1 and 975.9 ng/mL) most likely reflect dominantly classical sHLA-A, sHLA-B, and sHLA-C molecules. However, and paraphrasing Kessler et al,[22] characterization of specific HLA-A, HLA-B and HLA-C types will be crucial to optimally exploit the diagnostic value of sHLA class I molecules in dementia. On the other hand, the fact that 55 out of 56 elderly subjects were CMV seropositive points against the possibility that the differences found in the level of sHLA class I might have been caused by CMV infection, as previously reported for other immunological variables.[29] Conclusively, our findings provide a first insight into a new phenomenon but raises important questions to be answered. First, what would be the possible triggers for the release of HLA class I molecules into the extracellular milieu, and do they exist in cognitively impaired patients? Second, what are the physiological implications of the existence of circulating sHLA class I molecules in the context of neurodegeneration? Third, what is the relevance of having the HLA-A23 or HLA-A24 serotypes in the context of dementia? Fourth, is there a relationship between secretion of sHLA class I molecules and secretion of extracellular vesicles?

4.1 | Triggers for the release of HLA class I molecules

The presence of sHLA class I molecules in body fluids, namely plasma, is due to the constitutive process of internalization, intracellular trafficking, and recycling of cell surface HLA class I molecules that takes place under homeostatic conditions in nucleated cells, namely T cells and dendritic cells.[36,37] Under inflammatory conditions, a sizeable fraction of the internalized HLA class I molecules is not re-expressed at the cell surface but secreted into the extracellular milieu.[8] Indeed, during inflammation, cytokines such as IFN-ɣ play a key role in increasing HLA class I gene transcription, augmenting the expression of cell surface HLA class I molecules, and enhancing their secretion.[38–40] Considering these findings, it can be proposed that the increased levels of sHLA class I found in the cognitively impaired group are triggered by the activation of cells of the innate (e.g., monocytes, microglia, dendritic cells) and adaptive (e.g., T cells) branches of the immunological system in response to endogenous factors and/or antigens released locally (e.g., ApoE, β-amyloid, gangliosides, etc.) in the brain parenchyma, and eventually presented outside the CNS to T cells by dendritic cells. In this regard, it is important to note that we have recently shown a higher production of IFN-ɣ by peripheral blood T cells from cognitively impaired elderly activated in vitro.[28]

4.2 | Implications of circulating sHLA class I molecules for neurodegeneration

A body of evidence points to HLA class I *loci* as risk factors for the development of neurodegenerative disorders in humans.[41,42] However, the deleterious effect of HLA class I alleles on neurodegeneration pertains to some specificities, such as HLA-A2, HLA-A3, and HLA-B7,[15,43] but not to others, such as HLA-A1, which have been linked to a late onset of neurodegeneration.[12,13] Of note, in vitro studies performed during the past decades have shown that sHLA class I molecules are endowed with immunomodulatory properties, including the inhibition of cytotoxic CD8+ T cells and NK cells after binding to CD8 and to inhibitory NK receptors. Due to their capacity to inhibit innate and adaptive immune responses, sHLA class I molecules are considered tolerogenic factors.[8] In this regard, in vitro studies with cultured embryonic mouse retina explants and wild type thalamic explants, or thalami from transgenic mice whose neurons express high levels of H-2 class I, revealed that soluble H-2D molecules inhibited retina neurite growth.[18] Overall, these studies suggest that soluble HLA class I molecules could modulate effector immune cells and CNS parenchymal cells (e.g., neurons and microglia) expressing their ligands. Therefore, it is plausible to think that in elderly subjects developing dementia immunomodulatory mechanisms are elicited by the neurodegenerative process, which may include secretion of sHLA class I molecules by activated T cells, monocytes/microglia, and dendritic cells.

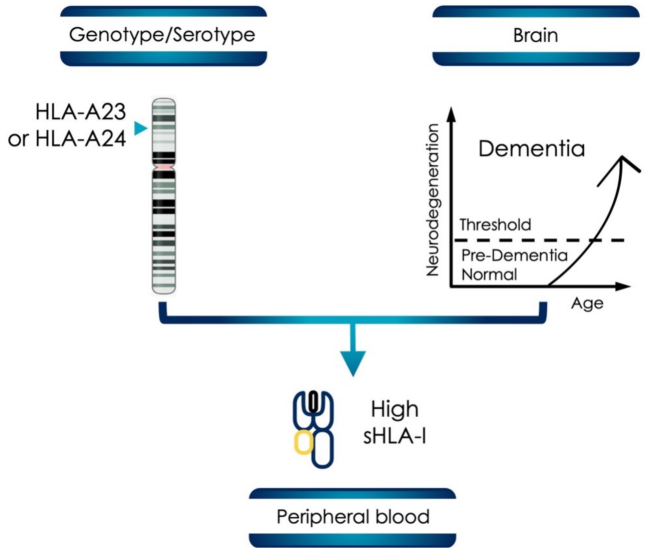

FIGURE 4 Proposed model illustrating how the HLA class I serotype/genotype interacts with the brain when the threshold between normality/pre-dementia and dementia is exceeded leading to an increased secretion of immunoregulatory sHLA class I molecules.

4.3 | Relevance of the HLA-A23 or HLA-A24 serotypes in the context of dementia

The results of this study have shown that elderly subjects carrying the HLA-A23/HLA-A24 serotypes (splits antigens of HLA-A9) have higher levels of sHLA class I than elderly subjects carrying other HLA class I serotypes (Figures 2 and 3). Our results are in accordance with earlier reports pointing to HLA-A23/HLA-A24 as high producers of sHLA class I molecules.[44] Noteworthy, recent studies have shown that the "high secreting" features appear to be due to epigenetic traits related to methylation patterns within the HLA-A promoter region that results in high expression mRNA levels of HLA-A23 and HLA-A24.[45] These data are most intriguing for several reasons. First, HLA-A23 and HLA-A24 molecules are ligands of KIR3DL1, a killer cell Ig-like family receptor member.[46] Second, KIR3DL1 alleles have the potential to inhibit the effector activities of NK cells and CD8+ T cells that express it, after interaction with HLA class I molecules containing the Bw4 epitope that is present on many HLA-B and some HLA-A alleles, including HLA-A23 and HLA-A24.[32,33] Third, KIR3DL1 alleles have been associated with protection from PD symptoms, implying a role for KIR3DL1-expressing cells in delaying PD progression.[47] Thus, in line with what was referred in Subsection 4.2, it can be proposed that the presence of higher levels of sHLA class I molecules (i.e., sHLA-A23 and sHLA-A24) in plasma of cognitively impaired elderly subjects with dementia could be part of the immunomodulatory mechanisms elicited by the ongoing neurodegenerative process (Figure 4).

4.4 | sHLA class I molecules and extracellular vesicles?

It is presently accepted that the pool of soluble HLA class I molecules found in plasma samples largely contain HLA molecules that are associated with extracellular vesicles and exosomes through their transmembrane domain.[48] Indeed, HLA class I molecules are an important part of the proteome of exosomes secreted by T cells and dendritic cells.[49,50] Although the scientific literature is conflicting regarding the exact role played by extracellular vesicles in inflammatory diseases, including neurodegenerative disorders, recent studies indicate that exosomes derived from CD8+ T cells and dendritic cells have immunosuppressor function that may contribute to downplay inflammatory responses.[51,52] In this context, whether the high levels of differentiated effector memory CD8+ T cells (CD8+ TEMRA) found in peripheral blood[29] and cerebrospinal fluid[53] of elderly subjects with cognitive impairment and dementia contribute to the pool of sHLA class I molecules and whether these molecules promote or try to prevent cognitive impairment are issues that warrant further investigations. In this respect, we have recently hypothesized that CD8+ TEMRA cells migrating into peripheral tissues, including the CNS, may fine-tune CNS homeostasis through the secretion of IFN-ɣ, TGF-β, and IL-10, and the interaction between HLA class I molecules and inhibitory NK receptors.[54] Interestingly, a recent multiomics study with peripheral blood samples from amnestic mild cognitive impairment (aMCI) patients has revealed that T cells with immunosuppressor function (without ascertaining whether CD4+ or CD8+, or both) represent a biomarker associated with aMCI.[55]

5 | CONCLUSIONS AND LIMITATIONS OF THE STUDY

This study has shown for the first time that the coexistence of HLA-A23/HLA-A24 and dementia is associated with high levels of plasma sHLA class I molecules, pointing toward sHLA class I as potential biomarkers for neurodegeneration in specific HLA class I carriers. This view is reinforced when considering that the HLA-A23 and HLA-A24 antigens carry the Bw4 epitope, a ligand of the inhibitory receptor KIR3DL1 expressed by NK cells and CD8+ TEMRA cells, and that the Bw4-KIR3DL1 interaction appears to suppress CD8+ T cell-mediated responses.[56,57] A similar scenario may be envisaged in neurodegenerative disorders, where neuronal antigen presentation may play a role in neuronal damage.[58] In summary, the obtained results provide a good foundation

for future studies with a larger cohort and age range and also looking into additional allele types not covered by the selected population.

AUTHOR CONTRIBUTIONS

Elsa M. Cardoso and Fernando A. Arosa conceived the study, analyzed data, and wrote the manuscript; Elsa M. Cardoso and Vânia Lourenço-Gomes performed the ELISA assays; André J. Esgalhado, Débora Reste-Ferreira and Nádia Oliveira performed blood samples collection, handling, and separation; Ana Saraiva Amaral coordinated the performance of cognitive tests to elderly subjects; Ignácio Verde coordinated the creation of the cohort of elderly subjects of the Beira Interior region (EBIcohort) and blood samples collection; António Martinho performed HLA serotyping; Jorge M.R. Gama and Elsa M. Cardoso performed statistical analysis; Olga Lourenço and Ana M. Fonseca provided scientific support; Rico Buchli provided scientific and technical support. All authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

Rico Buchli is affiliated with Pure Protein LLC, a company with financial interest to sell HLA proteins used within this study. All other authors confirm that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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