Antibodies against HSV-1 and Curli Show the Highest Correlation in Parkinson’s Disease Patients in Comparison to Healthy Controls

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1. Introduction

Parkinson’s disease (PD) is the second-most common neurodegenerative disease, with a prevalence of more than 1% in the population above 65 years of age which is projected to double by 2030 [1]. PD patients frequently suffer from motor dysfunctions,
including resting tremors, bradykinesia, rigidity, and gait abnormalities, as well as non-motor symptoms such as hyposmia, sleep disorders, depression, cognitive impairment (CI), and gastrointestinal (GI) symptoms [2–5].

PD is a multifactorial disorder resulting from a combination of genetic and environmental factors [5,6]. Over the past decade, it has become increasingly apparent that the gut microbiota and microbial pathogens may contribute to neurodegenerative diseases, either directly or through immune activation [7]. A recent study has shown that curli-producing bacteria in the gut microbiota could promote alpha-synuclein (α-syn) aggregation in both the GI tract and the brain, through immune system activation and/or cross-seeding (heterologous amyloid curli protein acts as a seed and facilitate the aggregation of α-syn) [7]. Thus, several bacteria and viruses have been associated with PD, including Herpes Simplex Virus 1 (HSV-1), Porphyromonas gingivalis (Pg), Mycobacterium avium subsp. paratuberculosis (MAP), Epstein–Barr virus (EBV), Cytomegalovirus (CMV), Helicobacter pylori (H. pylori), and Chlamydia pneumoniae (C. pneumoniae) [3,8–11].

The neuropathological changes in PD are defined by the degeneration of dopaminergic neurons, with the accumulation and aggregation of human amyloid protein α-syn to form Lewy bodies (LBs) in the brain and nervous system [12]. Moreover, α-syn deposits in the brain and enteric nervous system mediated by bacterial dysbiosis may happen years before the disease [13,14]. Interesting evidence has shown that the microbial amyloid curli is structurally similar to human amyloid α-syn, which is involved in PD [15]. Curli makes up as much as 85% of the extracellular matrix of enteric biofilms, which frequently contribute to cell–cell attachment and bacterial invasion in GI biofilms. This protein is expressed when enteric bacteria are grown under stressful environmental conditions [4,15].

The immune system recognizes both the bacterial amyloid curli and human amyloid utilizing the same receptors, such that bacterial amyloid also stimulates the immune system and induces inflammation [16,17] however, these bacterial amyloids can initiate additional α-syn deposits through cross-seeding, potentially indirectly causing neuroinflammation and then neurodegeneration [15].

A comprehensive demonstration of the role of the humoral immune response against bacterial amyloid and multiple pathogens in the PD pathogenesis is still lacking. Therefore, in this study, we aimed to evaluate the prevalence and magnitude of the immune response against immunogen peptides derived from human α-syn100–114 and bacterial amyloid curli (Curli133–141), Porphyromonas gingivalis, Pg (RgpA800–812, Kpg328–339), Aggregatibacter actinomycetemcomitans, Aa (LtxA1429–445, LtxA264–80), Mycobacterium avium subsp. paratuberculosis (MAP3865c125–133, MAP1,4-a-gbp157–173 and MAP_402718–32), Epstein–Barr virus (EBNA1400–413, BOLF1305–326), and Herpes Simplex Virus HSV-1 (UI4222–36) in PD patients compared with the general population.

2. Results

For this case–control study, we investigated a set of serum samples derived from 51 PD patients (27 females, 24 males; median ± SD: 74.05 ± 8.6) and compared their results with 58 HCs (30 females, 28 males; median ± SD: 72.5 ± 8.5). We found no statistically significant difference between the age and sex of PD patients compared to HCs (p = 0.346 and p > 0.99, respectively).

The intra- and inter-assay variation were 8.2–9.9% and 12.1–14.7%, respectively. Optical density values (OD) at 405 nm (OD405) were obtained from the IgG indirect-ELISA protocol with respect to 12 designed immunogenic peptides for all PD and HC sera, as demonstrated in Figure 1.
Figure 1. Analysis of Abs reactivity against human α-syn and pathogenic microorganism-derived peptides in PD patients and HCs. Serum samples were tested with indirect ELISA assay to plate-coated (A) α-syn100–114, (B) Curli133–141, (C) Kpg328–339, (D) RgpA800–812, (E) LtxA1429–445, (F) LtxA264–80, (G) UI4222–36, (H) EBNA1400–413, (I) BOLF1305–320, (J) MAP402718–32, (K) MAP1,4-α-gbp157–173, (L) MAP3865c125–133 peptides. The dotted lines represent the cut-off values calculated by ROC analysis; In the upper section of the graph, are indicated the Mann–Whitney p-value and the percentage of positive patients’ values calculated by the Fisher’s exact test. Statistical significant levels showed at p < 0.05, ns: not significant.

Based on our results, antibodies (Abs) against human amyloid-derived peptide (α-syn100–114) did not significantly different between PD patients and HCs (Figure 1A; p > 0.05), whereas Abs level against bacterial amyloid-derived peptide (Curli133–141) was significantly...
Based on our results, antibodies (Abs) against human amyloid-derived peptide (α-syn-100–114) were observed in 79.6% (40 out of 51) of PD patients (cut-off value 0.29; AUC = 0.902; $p$ < 0.0001; see Figure 1C). Concerning RgpA 800–812 peptide, we observed a significantly higher Abs response in PD patients (60.7%; 31 out of 51) than in HCs (24.1%; 14 out of 58) (cut-off value 0.29; AUC = 0.95; $p$ < 0.0001; see Figure 1D). The positivity and mean levels of anti- UI4222–36 Abs also showed a significant difference between PD patients (43.1%; 22 out of 51) than in HCs (25.8%; 15 out of 58) (cut-off value 0.25; $p$ < 0.0001; see Figure 1G).

The level of Abs against other immunogenic peptides (LtxA1429–445, LtxA2 64–80, EBNA1 400–413, BOLF1305–320, MAP4027 18–32, MAP1,4-a-gbp 157–173, MAP3865c 125–133 peptides) did not statistically differ between PD patients and HCs ($p > 0.05$).

Spearman’s correlation analysis was conducted to determine the possible correlations between OD values obtained against different immunogenic peptides between PD patients and HCs. The highest rate of correlation was observed between anti-UI4222–36 and anti-Curli 133–141 ($r = 0.811$, $p < 0.0001$), followed by anti-RgpA 800–812 and anti-Kpg 328–339 ($r = 0.659$, $p < 0.0001$), anti-LtxA1429–445 and anti-LtxA2 64–80 ($r = 0.653$, $p < 0.0001$), anti-BOLF1 305–320 and anti-LtxA1429–445 ($r = 0.513$, $p < 0.0001$), and anti-Curli 133–141 and anti-RgpA 800–812 ($r = 0.506$, $p < 0.0001$). Figure 2 shows the $r$ values obtained from the Spearman correlation analysis performed among the derived OD against the designed peptides.

![Figure 2](image)

Figure 2. Heatmap shows the $r$ values obtained from Spearman correlation analysis performed among derived peptides’ ODs.

In addition, a further Spearman correlation analysis was performed to evaluate a possible correlation between the severity of disease (HY scale 1 to 5) and ODs values derived from the indirect-ELISA assay. There were significant correlations between ODs
against LtxA\textsubscript{429–445} and the HY scale \((r = 0.306, p < 0.028)\), and between Kpg\textsubscript{328–339} and the HY scale \((r = 0.290, p < 0.038)\) in PD sera. There was no significant correlation between the OD values against other peptides and the HY scale.

3. Discussion

Parkinson’s Disease (PD) is a complex neurodegenerative amyloid disorder with unknown cause [1,6]. Growing evidence has demonstrated that the gut microbiota and microbial pathogens are involved in its etiology [7,18]. In addition, novel findings have emphasized that amyloid curli produced by Gram-negative enteric bacteria in the biofilm state in the GI tract has a link to neurodegenerative diseases [15].

In this study, Abs against human amyloid \(\alpha\)-syn and bacterial amyloid curli were investigated. We observed, for the first time, an increase in Abs level against bacterial amyloid curli in PD patients, compared to HCs \((p < 0.005)\), while no significant difference was observed for anti-human amyloid between the two groups. Several studies have reported no difference in serum human amyloid \(\alpha\)-syn Abs between patients with PD and HCs [19,20], consistent with our study. In contrast, several other studies have found high levels of \(\alpha\)-syn Abs in PD patients, compared to HC sera [21,22]. Future investigations are necessary to determine the \(\alpha\)-syn Abs level sub-classes in the different stages of PD, compared with HC for use as a therapeutic or diagnostic biomarker in PD patients.

Interestingly, in our study, the Abs against bacterial amyloid curli was significantly higher in PD patients than in HCs. The presence of Abs to a key biofilm component curli in 51% PD vs. 22.4% of HCs suggests that biofilm may have a potential role in the development of PD, possibly as cryptic reservoirs of \(\alpha\)-syn homolog curli. A recent finding has demonstrated that the presence of curli-expressing \(E.\ coli\) in mice microbiota increases \(\alpha\)-syn-mediated motor deficits and brain pathology [23]. A considerable correlation between persistent bacteriuria and anti-curli/eDNA IgG levels (IgGs against curli naturally complexed with bacterial extracellular DNA), detected in lupus and HC plasma, has been described in the study of Pachucki et al. [24]. In addition, IgA anti-curli/eDNA levels were higher in lupus donors, compared to controls [24]. However, detailed knowledge regarding the role of curli in the stimulation of the immune system and its relationship with PD requires further investigation. Moreover, this is the first report of anti-curli IgGs in PD patients, which could be a promising target for treatment and as a diagnostic biomarker in PD. Furthermore, we observed a higher prevalence of humoral response against peptides derived from periodontal pathogens \(P.\ gingivalis\) (Pg) and Aa in PD patients, compared to HCs, which was statistically meaningful for the anti-RgpA and Kpg IgG peptides. Periodontal pathologies are known to be linked to systemic inflammation [25], and \(P.\ gingivalis\) (Pg), especially, is associated with different systemic diseases, including PD, non-insulin-dependent diabetes mellitus [26,27], Alzheimer’s disease [28], rheumatoid arthritis [29,30], and cardiovascular disease [31,32]. These findings indicate the possible association between Pg and PD, confirming the hypothesis that Pg can induce a systemic antibody response, possibly influencing the progression of PD. On the other hand, there was a significant relationship between the increase in the level of antibodies against this bacterium and the severity of Parkinson’s disease (HY index). This relationship emphasizes the role played by oral infection during Parkinson’s disease. Despite various studies on the relationship of Pg with PD, no study was found on the relationship of Aa with PD. Díaz-Zúñiga et al. study showed that Aa can increase the risk of Alzheimer’s disease by specific inflammatory and immune responses in brain cells [33]. Therefore, the association between periodontal pathogens, especially Aa, in the progression of neurodegenerative diseases thus needs to be further investigated. It also seems that accurate oral and dental hygiene in Parkinson’s patients can be effective in prevention and reduction the symptoms of the disease. We observed a statistically significant difference in antibody levels against a common pathogen of the central nervous system, HSV-1 (Ul42\textsubscript{22–36}) in PD patients in comparison to HCs [9], which was consistent with other studies [34]. Furthermore, the antibodies able to recognize the HSV-1-Ul42\textsubscript{22–36} peptide are able to cross-react with the homologous human
α-syn_{100–114} epitope [9]. In this study, we highlight a positive correlation between Abs against HSV-1 and curli, supporting the hypothesis that HSV-1 infection may change the composition of the gut microbiota, which may lead to dysbiosis. The results from the study of Ramakrishna et al. showed that HSV and acyclovir can disrupt the gut bacterial community in a sex-biased manner in a C57BL/6 mice model [35]. In our study, the level of Abs against EBV peptides was higher in PD patients than in the healthy group; however, this difference was not statistically significant. Epidemiological studies have demonstrated that PD patients are significantly more Abs seropositive for EBV than HCs [36,37]. Latent EBV infection can trigger autoantibodies that can cross-react with α-syn and elevate α-syn aggregation [36,37]. Considering that EBV is one of several proposed environmental factors associated with PD, our study population is probably influenced by other genetic and environmental factors. On the other hand, to obtain more accurate epidemiological statistical results, it is suggested to investigate of Abs against other EBV-immunogenic peptides in a larger number of PD patients and comparison with HC, in future studies. As has been reported in a previous study, a high level of Ab-mediated immune reaction was detected against MAP3865c_{207–219}, and MAP3865c_{82–97} peptides, while no significant reaction was observed against MAP3865c_{81–95} and MAP3865c_{44–59} peptides [8]. In this study, Abs levels against other MAP epitopes were the same in the two populations (i.e., PD and HCs).

The most obvious limitation of the current study was that of small sample size for the evaluation of Abs against these peptides. Moreover, selection of other microbial peptides with broad immunogenic potential designed from these organisms and checking Abs against them in large sample sizes is recommended.

4. Materials and Methods

4.1. Study Population and Blood Collection

For the current case–control study, we examined two populations, PD patients, and healthy controls (HCs) during the period between July 2021 and August 2022. This study was approved by the Ethics Committee of the University of Sassari in 2019 (prot 2159/CE). Informed consent was obtained from all individual participants. All patients were diagnosed based on medical history, clinical symptoms and neurological and physical examination. All data on age, gender, and HY scale (Hoehn and Yahr Scale) were retrieved from patient records. During the same period, healthy controls, with no personal or familial history of diagnostic PD, whose age and gender matched with those of the patients, were included in the study as controls. Blood samples were collected from participants referred to the Parkinson Institute hospital at the Azienda Ospedaliera Universitaria of Sassari. Then, sera were separated according to the standard method [38] and preserved at –80 °C in a freezer.

4.2. Peptides

Synthesis of an immunogenic peptide derived from bacterial amyloid curli (Curli_{133–141}: NSSVNVTQV) was designed using the Immune Epitope Database and Analysis Resource (IEDB) and synthesized at >95% purity (LifeTein, South Plainfield, NJ, USA).

Immunogenic peptides derived from human amyloid (α-syn_{100–114}), Porphyromonas gingivalis, Pg (RgpA_{800–812}, Kp_{832–839}), Aggregatibacter actinomycetemcomitans (LtxA1_{429–445}, LtxA2_{64–80}), Mycobacterium avium subsp. paratuberculosis (MAP3865c_{125–133}, MAP1,4-a-gbp_{157–173} and MAP_4027_{18–32}), Epstein–Barr virus (EBNA1_{400–413}, BOLF1_{305–320}), and Herpes Simplex Virus 1 (UI422_{22–26}) were selected from peptides used in previous studies [9,18,30,39]. All peptides were re-suspended in dimethyl sulfoxide (DMSO) at a final concentration of 10 µg/mL and stored at –80 °C until further use (Table 1).
Table 1. Immunogenic peptides used as antigens in the ELISA assay.

| Epitope       | Epitope Sequence     | Epitope Position |
|---------------|----------------------|------------------|
| α-syn100-114  | LGKNEEGAPQEGILE      | 100–114          |
| Curli133-141  | NSSVNVTVQ           | 133–141          |
| UI4222-36     | LGQPEEGAPCQVVLQ      | 22–36            |
| RgpA800-812   | ADPVVTIINIVT         | 800–812          |
| Kpg328-339    | VTDLLYSAVGDG         | 328–339          |
| LtxA1429-445  | AWENKYGKNTFENGYDA    | 429–445          |
| LtxA264-80    | TALIKAQKLGIETYHE     | 64–80            |
| MAP3865c125-133 | MIAVALAGL          | 125–133          |
| MAP1,4-a-gbp157-173 | GTVELLGGPLAHFQQL  | 157–173          |
| MAP_402718-32  | AVVPLAYAAARL        | 18–32            |
| EBNA1400-413  | PGRRPFHPVGEAD       | 400–413          |
| BOLF1305-320  | AAVPVLAFAARLRLLE    | 305–320          |

4.3. Enzyme-Linked Immunosorbent Assay (ELISA)

Indirect ELISA was performed to investigate the specific IgG antibodies against the designed peptides mentioned in the study. In brief, 50 µL of each peptide at a concentration of 10 µg/mL in 0.05 M carbonate/bicarbonate buffer, at pH 9.5 (Sigma-Aldrich, St. Louis, MO, USA), were coated in 96-well plates (Thermo Fisher Scientific, South San Francisco, CA, USA) and incubated at 4 °C for 1 day. The coating solution on plates was removed and blotted on paper towels. Plates were incubated for one hour at room temperature (RT) in a blocking solution with 200 µL of 5% non-fat dried milk (5 g non-fat dried milk powder in 100 mL 1× PBS; Sigma-Aldrich, St. Louis, MO, USA) and washed twice in a solution with 0.05% Tween-20 and phosphate-buffered saline (1× PBS-T; Sigma-Aldrich, St. Louis, MO, USA). Plasma samples (diluted 1:100; 1 µL plasma to 99 µL 1× PBS-T) were added, and the plates were incubated for 2 h at RT. Then, each plate was washed five times in 1× PBS-T and incubated for one hour at RT with 100 µL of PBS and anti-human IgG alkaline phosphatase conjugated antibody produced in goat (1:1000; Sigma-Aldrich, St. Louis, MO, USA). Plates were washed five times in 1× PBS-T and incubated in a dark environment for eight to ten minutes in milli-Q water and p-nitrophenyl phosphate (One p-290 nitrophenyl phosphate tablet and one Tris buffer tablet were dissolved in 20 µL of milli-Q water; Sigma-Aldrich, St. Louis, MO, USA), and the optical density (OD) was read at a wavelength of 405 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). All samples were repeated in duplicate, and positive controls were used for each peptide. The positive sample was a sample previously tested for strong reactivity to the selected peptides, and not reactivity to irrelevant peptides, in order to verify the binding specificity. The negative controls were samples from patients previously tested for the same peptides which had a weak reaction. Moreover, a technical negative control was added, where no sera was added into the peptide-coated wells. The OD values were normalized to a highly positive control serum with absorbance reactivity set at 1.0 OD. Results are expressed as means of duplicate 405 nm OD values.

Intra-assay variation was calculated based on the mean of the CV percentages (%CVs) obtained from OD measurements repeated two times for each serum in the same plate. Inter-assay variation was calculated based on the mean of %CVs obtained from experiments repeated two times for each serum in two separate plates on two different days. Inter-assay variation was done for 30 serum samples with high, low, and moderate ODs.
4.4. Statistical Analysis

The analysis was performed using GraphPad Prism version 8.0 software (San Diego, CA, USA). The data distribution was analyzed using the D’Agostino–Pearson omnibus normality test and, consequently, the Shapiro–Wilk test. Non-parametric data were analyzed using the Mann–Whitney U test to compare Abs against different peptides in PD patients compared to HCs. Student’s t-test and Fisher’s exact test were applied to compare the matching of age and sex in PD patients with the HCs group. A value of \( p < 0.05 \) was considered significant. Optimal cut-off points to discriminate between positive and negative samples were identified based on the receiver operating characteristic (ROC) curve with \( \geq 90\% \) specificity and 95% confidence interval. In addition, Fisher’s exact test was employed to compare the percentages of positive subjects in the two groups. The correlation between OD values obtained by the ELISA test from different peptides was explored through bivariate correlation and regression analysis using the Stata software. In addition, the correlation analysis between the HY scale and OD values obtained by the ELISA test from different peptides was explored through bivariate correlation and regression analysis.

5. Conclusions

In this study, we reported a significantly increased humoral response against curli, Pg, and HSV-1 in PD patients, thus implying the important role of these factors in the pathogenesis of the disease. Therefore, while the development of PD has not yet been associated with unique microbial species and their products, more studies will be necessary to examine the potential interactions between the bacterial amyloid curli and the human amyloid, in order to understand their relevance in the pathogenesis of PD. In addition, a better comprehension of the intricate relationship between microbial pathogens and PD may help in the future to develop effective strategies to detection and preventing the development of the PD.

Author Contributions: L.A.S. conceived and designed the study, and revised the final version of the manuscript; K.P. recruited PD patients and healthy controls; S.J. drafted the manuscript and carried out the experiments; M.N., E.R.S., and S.R. performed the experiments and analyzed the data statistically. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data that support the finding of this study are available from the corresponding author, upon reasonable request.

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Conflicts of Interest: The authors declare, that they have no conflict of interest.

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