

Peer Review Information

Journal: Nature Structural and Molecular Biology

Manuscript Title: Global analysis of the structural proteome in Parkinson's disease patients to identify a new class of biomarker

Corresponding author name(s): Dr Paola Picotti

Editorial Notes:

Redactions – unpublished data

Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Redactions – confidential patient information

Parts of this Peer Review File have been redacted as indicated to maintain patient confidentiality.

Redactions – published data

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

Redactions – reviewer opt-out

Parts of this Peer Review File have been redacted as indicated as we could not obtain permission to publish the reports of reviewer no. **XX**.

Reviewer comments in marked-up manuscript

In their review of the [first/second/third/...] version of this manuscript, reviewer no. **XX** added their comments to the manuscript file. These comments, excluding minor textual revisions, have been copied into this Peer Review File.

Reviewer Comments & Decisions:

Decision Letter, initial version:
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21st Jun 2021

Dear Paola,

Thank you again for submitting your manuscript "Global analysis of protein structural variation identifies predictors for Parkinson's Disease". My apologies once more for the delay in reaching a decision, but we have received the full set of reviewer comments (appended below) only at the end of last week. In light of these reports, we remain interested in your study and we would like to invite you to respond to the comments of the referees, in the form of a revised manuscript.

You will see that while the reviewers appreciate the approach and find the data set valuable, they also raise quite substantial concerns that will need to be addressed in a revision. Specifically, both reviewer #1 and #3 request validation of the top marker proteins in an independent cohort of PD patients, which, according to reviewer #3, is standard in the field. This would be required for further consideration of the study here at NSMB, unless the focus of the manuscript is shifted from PD biomarkers by removing claims towards functionally following-up on the potential disease relevance of a small subset of identified structural variations.

In addition, reviewer #1 and #2 would like to see at least some independent validation of the observed structural changes and the suggested mechanism and functional consequences for a select subset of proteins. We agree that this should be included in the manuscript.

Assessing SCF form genetic versus idiopathic forms of PD (reviewer #1) or examining the relationship between protein structural changes in low versus high oligomeric/total alpha-synuclein PD groups (reviewer #3) won't be required, unless the data is already available for the current cohort. The concerns raised by reviewer #3 regarding the feasibility of this type of MS analysis in the clinic or its application to brain tissue, while valid, will not be deciding factors in our editorial decisions.

Please do not hesitate to contact me if you would like to discuss these points and the required revisions. We are committed to providing a fair and constructive peer-review process; if there are requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome, we would be happy to discuss these further.

Once you are ready to submit the revised manuscript, please be sure to address/respond to all concerns of the referees in full in a point-by-point response and highlight all changes in the revised manuscript text file.

We appreciate the requested revisions are extensive. We thus expect to see your revised manuscript within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision as long as nothing similar has been accepted for publication at NSMB or published elsewhere. Should your manuscript be substantially delayed without notifying us in advance and your article is eventually published, the received date would be that of the revised, not the original, version.

As you already know, we put great emphasis on ensuring that the methods and statistics reported in our papers are correct and accurate. As such, if there are any changes that should be reported, please submit an updated version of the Reporting Summary along with your revision.

Reporting Summary:

<https://www.nature.com/documents/nr-reporting-summary.pdf>

Please note that the form is a dynamic 'smart pdf' and must therefore be downloaded and completed in Adobe Reader.

When submitting the revised version of your manuscript, please pay close attention to our [href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines](https://www.nature.com/nature-research/editorial-policies/image-integrity) and to the following points below:

- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
- that control panels for gels and western blots are appropriately described as loading on sample processing controls
- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

Please note that all key data shown in the main figures as cropped gels or blots should be presented in uncropped form, with molecular weight markers. These data can be aggregated into a single supplementary figure. While these data can be displayed in a relatively informal style, they must refer back to the relevant figures. These data should be submitted with the last revision, prior to acceptance, but you may want to start putting it together at this point.

SOURCE DATA: we also urge authors to provide, in tabular form, the data underlying the graphical representations used in figures. This is to further increase transparency in data reporting, as detailed in this editorial (<http://www.nature.com/nsmb/journal/v22/n10/full/nsmb.3110.html>). Spreadsheets can be submitted in excel format. Only one (1) file per figure is permitted; thus, for multi-paneled figures, the source data for each panel should be clearly labeled in the Excel file; alternately the data can be provided as multiple, clearly labeled sheets in an Excel file. When submitting files, the title field should indicate which figure the source data pertains to. We encourage our authors to provide source data at the revision stage, so that they are part of the peer-review process.

Mandatory deposition of data is required for certain data types. Please find the complete NRG policies on data availability at <http://www.nature.com/authors/policies/availability.html>.

While we encourage the use of color in preparing figures, please note that this will incur a charge to partially defray the cost of printing. Information about color charges can be found at <http://www.nature.com/nsmb/authors/submit/index.html#costs>

Nature Structural & Molecular Biology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. This applies to primary research papers only. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit www.springernature.com/orcid.

Please use the link below to submit your revised manuscript and related files:

[Redacted]

Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

With kind regards,
Anke

Anke Sparmann, PhD
Senior Editor
Nature Structural and Molecular Biology
ORCID 0000-0001-7695-2049

Referee expertise:

Referee #1: Molecular mechanism of PD

Referee #2: Mass-spectrometry

Referee #3: PD biomarkers

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

This is a very interesting study by a group with a strong track record in the field of 3D proteomics. The study is original and focuses on the assessment of protein structural variation in the CSF of a control cohort and in a cohort of Parkinson's disease patients. The authors report the identification of 76 proteins as structurally altered in the CSF of PD patients.

The type of study is extremely valuable and promising for the identification of biomarkers for PD and for other synucleinopathies.

However, I have some important concerns about the actual significance and impact of the findings:

- A major concern is that the study was not validated in an independent cohort of PD patients. In order to have confidence in the set of proteins identified as discriminating PD from healthy individuals, it would be essential to assess an independent cohort of patients.
- It would be extremely interesting to assess CSF from genetic (e.g. LRRK2) vs idiopathic forms of PD.
- In addition to a-synuclein, did the authors search for the presence of other proteins associated with PD?
- Given the important role a-synuclein plays in PD, the authors looked for this protein in their analysis. Given the low levels in CSF, they could not detect the protein. Therefore, I find it unnecessary to further discuss and assess the connection to a-synuclein levels, as this becomes confusing and difficult

to reconcile.

- Although the modelling performed is interesting, one would expect to see some validation studies to confirm the significance and biological relevance of the proteins/pathways identified.

Minor comments:

- On page 3, the authors say the control group had 51 healthy individuals. On page 5, they probably made a mistake and mention 52 healthy individuals... so this should be corrected.

- Figure numbers should be verified as some are repeated and some figures seem out of the correct order.

- I could not see Figure 5.

- Why is the Methods section separate from the text, with a separate list of references? Is this a requirement from the journal?

Reviewer #2:

Remarks to the Author:

In the manuscript 'Global analysis of protein structural variation identifies predictors for Parkinson's Disease' Picotti and colleagues present an exciting extension of the scope of their limited proteolysis approach (Lip-MS) to biomarker discovery for Parkinson's disease.

The manuscript describes a proof-of-principle study demonstrating technical feasibility and highlighting exciting new opportunities for protein biomarker discovery in cerebrospinal fluid by including low resolution structural characteristics. LIP-MS was applied to characterize the CSF of early diagnosed PD patients relative to controls and a total of 88 informative peptides corresponding to 76 proteins were identified as differential suggesting structural alterations in the CSF of PD patients. Structural features were found to perform better for patient classification than protein abundance features alone, albeit somewhat weaker than the α -synuclein gold standard.

The manuscript is well written and the method and its application will be of interest for the broad readership of this journal as well as for researchers interested in neurodegenerative disease and protein biomarkers. Whilst it is understandable that the origin and molecular cause of observed distinct structural features cannot be plausibly explained or investigated in each case, the study as presented to date lacks validation and it is recommended to follow-up on a select few observations using independent methods when revising the manuscript (see also the detailed points below).

Major:

- Healthy and diseased cohorts have marked differences in gender bias. This is sought to be compensated by linear models, but no evidence is provided how well these work. Figure 2 (should be 3) c indicates that the model may overcompensate for sex as low pvalue findings seem underrepresented?

- The bimodality described for the Fructose-bisphosphate aldolase peptide ALQASALK is difficult to understand as the proposed distinct structural states would need to exclusively apply to individuals in the cohorts – e.g. engaged with substrate or not.

Do other parameters such as sex or batch effects correlate with the observed lip-intensity clusters? I wonder if simple limited proteolysis experiments with recombinant enzyme in absence or presence of substrate would not be a convenient and convincing means to underpin the finding and its structural interpretation.

- Given the resource character of the manuscript, data descriptions in the supplementary material should be more comprehensive and self-explanatory. For example the header of supplementary table

5 states: LIP peptide intensities included in the analysis of structural variation. However, there is no column that lists peptide intensities, instead a coefficient and a pvalue are listed. It is quite cumbersome to figure out how these were calculated as there is no cross-referencing between supplementary material and materials and methods.

- Please avoid the term unbiased in the abstract and elsewhere when referring to LIP-MS, as in any other proteomics method there are several biases in detection related to abundance of the protein of interest and physico-chemical properties of the LIP peptides etc. The approach certainly enables the hypothesis-free and untargeted detection of markers but it is not unbiased.

- An external benchmark how well protein abundance measurements in CSF inform on PD status would be desirable. How well do protein abundance findings in this study correlate with previous studies? Are the structure-based markers fully novel or just distinct from what was found on protein abundance levels in this study? Relevant prior art may include:

<https://www.nature.com/articles/s41598-020-59414-4>

- A total of 76 proteins were identified as containing differential structural features in PD vs healthy control samples. For a small subset of these interesting discussion is provided how structural changes in these proteins might be linked to mechanisms relevant for disease pathology. However, not in a single case the structural changes were validated through independent means, nor the cause of the observed structural change is identified. E.g. substrate occupancy, dependent variations in PSAT1 should be detectable with other means or on recombinant protein and may inform whether the observed differential LIP-peptides are due to differences in occupancy or may be caused by other mechanisms that may ultimately lead to altered PSAT1 activity.

- Classification characteristics of the models using structural changes are quite promising, my only concern is that the argumentation that this cannot be due to overfitting (lines 425 ff) is vague and not supported by independent data or analyses.

Minor:

- A few more introductory words on the LIP-MS approach would be valuable for the broader readership of this journal. Could be included in the legend of Figure 1 if otherwise less feasible.

- In times it can be difficult to follow how different experimental constraints led to the numbers of proteins and peptides discussed. A supplementary table detailing the numbers of proteins and peptides identified in CSF and clarifying how many of these were informative in LIP-MS etc. would be useful.

- Figure 2 is labeled as Figure 1 in the manuscript and similarly Figure 2 should be Figure 3 and Figure 5 is labeled as Figure 3. I would recommend carefully checking all references to figures, tables, and other supplementary material.

- P7, please reference to a supplementary table where all 13 bimodal highly variable peptides are highlighted. Do they behave similar to the fructose-bisphosphate aldolase or rather like semaphorin?

Reviewer #3:

Remarks to the Author:

This novel study uses limited proteolysis-mass spectrometry (LiP-MS) for the unbiased detection of global protein structural changes in the CSF of 52 Parkinson's disease (PD) patients in comparison to 51 age-matched healthy controls (HCs). Authors identified 76 proteins as structurally altered, several which have been previously associated with PD (GO enrichment analysis). They were able to disentangle the structural variation from those that occur due to protein abundance variation and showed that the prior is much more significantly associated with the disease status. These structural changes in PD vs. HC were dependent on age but not gender. They also examined the protein

structural variability in the CSF of HCs and showed that highly variable peptides had a lower propensity to form beta strands and a higher propensity to form alpha-helices, greater propensity for protein-protein interactions and higher disorder compared to non-variable peptides. This is a useful resource as these highly variable CSF proteins across healthy individuals are unlikely to become good biomarker candidates.

The structurally varied proteins included enrichment of those that have been clearly implicated in the pathogenesis of PD e.g. synaptic proteins, vitamin D, and thus the analysis is mechanistically interesting and valuable. However, it is very difficult to see how this type of expensive and laborious analysis could be taken forward as clinically useful biomarker. It is unlikely that PD CSF samples could routinely be assessed by MS, and there is no information how well this MS analysis could stratify them from other synucleinopathies e.g. multiple system atrophy or even other neurodegenerative disorders e.g. Alzheimer's disease. Furthermore, there is no validation of their top structurally varied proteins in an independent cohort, which should be a standard for this type of analysis. It is also disappointing that alpha-synuclein peptides were not detected in the MS analysis due to their low endogenous levels as this obviously would have been very informative information. Authors compared the predictive value of five LiP peptides to oligomeric/total alpha-synuclein ELISA levels that performed together improved the accuracy compared to one marker alone. I think it would have been more interesting to examine the relationship between protein structural changes in low versus high oligomeric/total alpha-synuclein PD groups, as this could reveal something about the possible binding partner changes. I also agree with the authors that the this MS analysis should be applied to the brain tissue, where it may be more useful as a research tool to reveal more directly the structural and functional changes related to disease pathology.

Minor remarks:

Please include some references to numerous recent studies that have shown alpha-synuclein seeding assays to have a very good sensitivity and specificity for PD patients.

There are two figures labelled as Figure 1, please correct.

Can you please add any statistical significant differences between the groups to Suppl. Fig 1A? Was the gender different between PD and HC groups?

Author Rebuttal to Initial comments
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Summary for editor and all referees.

We thank the referees and the editor for the constructive comments. Here we summarize the main revisions.

1. We have added structural proteomic data on PD brain samples.

To address the pathological relevance of structural changes observed in the CSF of Parkinson's patients and potentially corroborate some of them in another tissue and different set of samples, we generated new structural proteomics data from brain tissue samples from a small independent cohort consisting of 5 PD patients and 5 healthy individuals. This also extends our analysis from biofluids to solid tissues. We report the following findings from this analysis:

- As we previously observed in the CSF, peptides that reflect structural changes in the PD brain are much more strongly correlated with disease status than are peptides that reflect protein abundance changes or other, protease-independent changes (e.g., PTMs or peptide sequence changes). We show these results in Figure 3F in the revised manuscript. This corroborates the main finding of our paper that global structural analyses are more sensitive than classical proteomics data in detecting pathological alterations.
- Further, out of the 76 proteins that we reported to change structurally in the CSF of PD patients versus healthy individuals, we detected 27 proteins in the brain data set. Note that the overall overlap between proteins detected in brain and CSF is not expected to be large (we detected 208 overlapping proteins in total in both sample types) given that these are quite different sample types. Strikingly, 16/27 (60%) of these proteins show a structural change between PD and healthy individuals in brain as well as in CSF. Further, about half of these proteins have strong links to PD based on the literature, including synaptotagmin 1 (SYT1) which regulates dopamine release, peroxiredoxin 6 (PRDX6), a known Lewy Body component, and stathmin 2 (STMN2) for which knockdown recapitulates several PD-like phenotypes in the mouse. We list all the PD-linked proteins that change structurally in both brain and CSF in Supplementary Table 8 and show some of these examples in Figure 4 and Extended Data Figure 4 in the revised manuscript.
- In total we detect 755 peptides in both brain and CSF, most of which do not change in PD. A small fraction of these (11/755) are structural peptides i.e., indicate structural changes in the CSF of PD compared to healthy individuals. Notably, most of these peptides (8/11) change their intensity in the same direction in the brain as they do in the CSF, which is very unlikely to have occurred by chance, and indicates that these specific structural changes are conserved between brain and CSF. We show these results in Extended Data Figure 3C and specific examples in Figure 4 and Extended Data Figure 4.

Taken together, these results provide strong support that the structural changes we detect in the CSF reflect changes in the brain, in an independent set of patients, and that the structural signal is informative about disease processes. The data also further validate our central argument - that a global structural analysis is a powerful approach to identify proteomic changes that are qualitatively different from abundance effects between the healthy and disease states and could be a valuable source of disease biomarkers in the future.

2. Emphasis on the new concept rather than practical biomarkers.

We have revised the manuscript to clarify our aim to present a new *concept* of biomarker discovery via a global protein structural analysis. This is an entirely new concept and as such, this manuscript is quite different from a more standard biomarker discovery pipeline. We entirely agree that much more work is needed before multidimensional structural signatures can form the basis for clinical tests. To reflect that and to address the reviewer's concerns, we have toned down statements about the identification of candidate biomarkers. We now put more emphasis on our new concept rather than on specific new biomarker candidates. We have changed the title of the manuscript to reflect this, made explicit in the abstract and the introduction that our study is intended to introduce a new concept and does not include an independent validation, and have added substantial discussion on the challenges and hurdles any translational effort will face.

Referees

Reviewer #1:

Remarks to the Author: This is a very interesting study by a group with a strong track record in the field of 3D proteomics. The study is original and focuses on the assessment of protein structural variation in the CSF of a control cohort and in a cohort of Parkinson's disease patients. The authors report the identification of 76 proteins as structurally altered in the CSF of PD patients. The type of study is extremely valuable and promising for the identification of biomarkers for PD and for other synucleinopathies.

We thank the reviewer for the encouraging comments.

However, I have some important concerns about the actual significance and impact of the findings:

- A major concern is that the study was not validated in an independent cohort of PD patients. In order to have confidence in the set of proteins identified as discriminating PD from healthy individuals, it would be essential to assess an independent cohort of patients.

We agree that validation of the structural signal in independent patient cohorts is an important next step. However, assembling the cohorts required for an independent validation effort, in particular CSF from healthy controls, is a substantial and long-term effort that is out of the scope and timeline of this proof-of-principle discovery study. We are in the process of assembling this cohort (see also response to referee 3 for more details).

In response to this criticism, we have made the following changes to the revised manuscript:

(i) As discussed also in the summary section above, we have now done a global structural analysis of postmortem brain tissue from a small, independent set of PD patients (n=5) and healthy individuals (n=5). Out of the 76 proteins that we reported to change structurally in the CSF of PD patients versus healthy individuals, we detected 27 proteins in the brain data set. Note that the overall overlap between proteins in brain and CSF cannot be too large (208 proteins detected in both types of samples), as they are quite different sample types. Strikingly, 16/27 of these proteins show a structural change between PD and healthy individuals in brain as well as in CSF. Further, about half of these proteins show strong links to PD based on current literature. Finally, 11 peptides that indicate a structural change between PD and healthy in CSF are also detected in the brain dataset; the majority (8/11) of these peptides change in the same direction between PD and healthy individuals in both brain and CSF. Together, these data provide strong support that the structural changes we detect in the CSF are recapitulated in another tissue that is more closely related to pathology, in an independent set of patients, and that the structural signal is informative about disease processes.

(ii) We have emphasized the conceptual nature of our study and the need for extension to independent cohorts and toned down the identification of specific biomarker candidates.

(iii) We have added discussion on the need for validation and on how the structural changes we uncover could be used as the basis for clinical assays based on conformational antibodies, a potential path to clinical use that has precedent in other diseases; we have also added discussion on the hurdles inherent in translational efforts.

- It would be extremely interesting to assess CSF from genetic (e.g., LRRK2) vs idiopathic forms of PD.

We agree that this would be an interesting study. However, our study cohort explicitly excluded known genetic forms of PD, and these forms are therefore not the focus of this work. We have now made the exclusion of known familial PD clearer in the methods section and the results section of the manuscript.

- In addition to α -synuclein, did the authors search for the presence of other proteins associated with PD?

We have now compared our lists of structurally altered proteins in brain and in CSF with genes linked to PD based on the NHGRI-EBI catalogue of genome-wide association studies (GWAS). Of the 76 proteins that show structural changes in the CSF, 2 correspond to PD-linked genes in GWAS, these are the α -1 (III) chain of collagen (COL3A1) and a peptidyl-glycine α -amidating monooxygenase (PAM). Of the 334 proteins that show structural changes in the brain, 3 correspond

to PD-linked genes in GWAS, these are AP2-associated protein kinase 1 (AAK1), CYFIP-related Rac1 interactor B (CYRIB) and Apolipoprotein E (APOE). The latter is consistent with ApoE being a known component of Lewy Bodies in PD brains. We have reported these associations in the revised manuscript. Further, we have conducted a literature analysis for proteins that change both in the brain and in CSF and found a number of other strong links to PD mechanisms; this is discussed in the revised manuscript and summarized in Supplementary Table 8.

- Given the important role *a*-synuclein plays in PD, the authors looked for this protein in their analysis. Given the low levels in CSF, they could not detect the protein. Therefore, I find it unnecessary to further discuss and assess the connection to *a*-synuclein levels, as this becomes confusing and difficult to reconcile.

We understand the point, but do not fully agree. For the CSF data, where *a*Syn could not be detected, we compared the performance of our new structural signal to the ratio of oligomeric to total *a*Syn as the most promising, previously suggested potential biomarker. Our goal was simply to assess these structural peptides, despite their preliminary nature, as potential biomarkers. We find it encouraging that the structural signal from this small discovery study, with still much room for technical optimization, is close to that of the most promising potential biomarker, which is based on technically optimized ELISAs. We continue to think this is useful information to convey to the community, so we have retained these data in the revised paper but have tried to make our reasoning clearer in the text. We note as well that referee 3 was interested in potential differences between the low and high oligomer/total *a*Syn ratio groups, likely reflecting ongoing community interest in potential PD subsets.

Also, unlike in the CSF, *a*Syn is abundant in the brain and is thus readily detected in our newly added data on brain samples. The data show evidence for a structural change between PD patients and healthy individuals but this does not reach statistical significance due to an outlier individual. Out of 20 detected *a*Syn structural peptides, 3 peptides show a significant difference between healthy and PD samples, when the outlier is removed (Figure R1); we expect that data on a larger cohort will help resolve this issue. We have mentioned these results in the revised manuscript but do not show the data as we do not see an overall statistically significant change, and as *a*Syn is well known to be associated with PD.

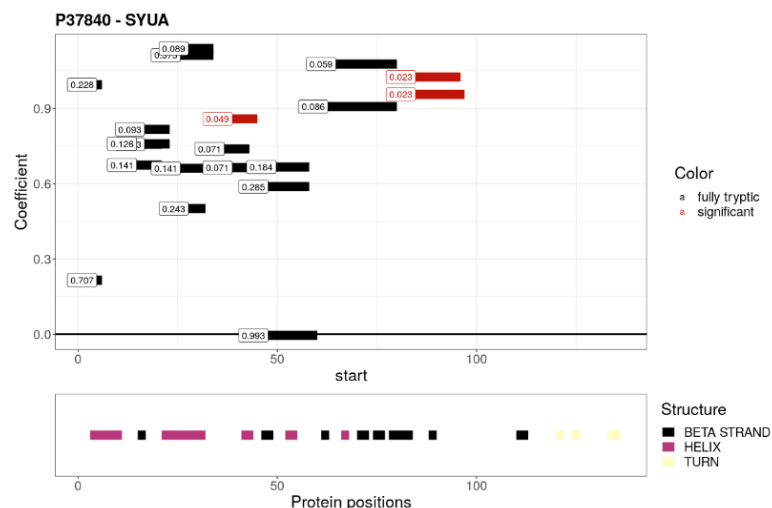


Figure R1. Structural analysis of *a*Syn in brain samples. The plot shows all detected peptides mapped along the *a*Syn linear sequence. Red indicates peptides that significantly change between healthy and PD brain ($p < 0.05$; P-values were calculated after removal of the outlier individual).

- Although the modelling performed is interesting, one would expect to see some validation studies to confirm the significance and biological relevance of the proteins/pathways identified.

Please see our earlier response and the summary at the beginning of this document, describing newly added data comparing post-mortem brain samples of PD patients and healthy individuals. We now validate that several of the proteins and even some specific peptides that change structure in PD CSF also do so in PD brain samples in a small independent cohort. Several of these proteins are clearly linked to PD, thus strongly supporting the biological- and disease-relevance of hits from our

global structural analyses. These new data are shown in Figure 3F, Figure 4, and Extended Data Figure 4.

We have also attempted several *in vitro* experiments testing hypotheses about the specific structural changes underlying our global analysis. We thank the referee for this suggestion as it has helped us both support and refute specific hypotheses. We note that conclusive validation would require high-resolution structural studies compatible with *in situ*/complex samples, which are out of the scope of this study.

First, we used LiP-MS on purified vitamin D-binding protein *in vitro*, to ask whether the single structural change we had observed in PD versus healthy CSF (Figure R2, left) might reflect substrate binding. We incubated purified Vitamin D-binding protein with its substrate (calcitriol) in various molar ratios and compared the LiP-MS patterns with and without substrate. For the condition with a 3.3X molar ratio (substrate:protein), the *in situ*-measured structural peptide also changed upon substrate addition *in vitro* (Figure R2, right). Other peptides, which map to the substrate binding site, show an even stronger fold change upon substrate addition, but these regions were in part not covered in the *in situ* experiment. These data are therefore consistent with a structural change that occurs upon vitamin D binding, although they are not complete due to lack of coverage *in situ*. We now show these data in Figure 4 and state in the manuscript that further studies are needed.

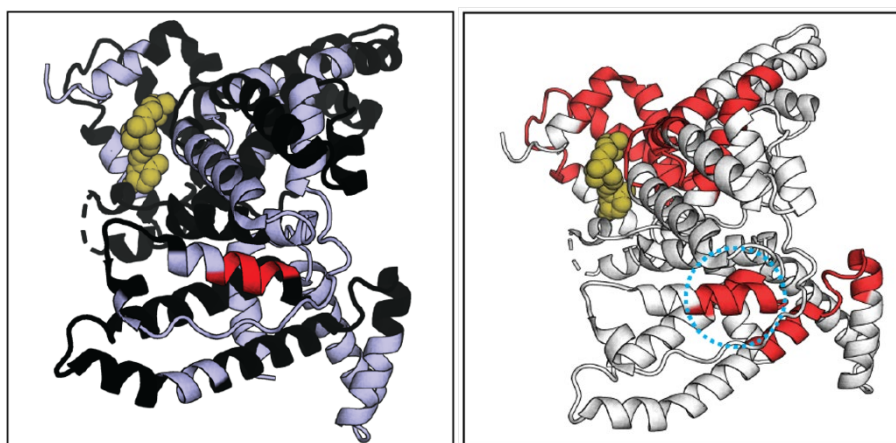


Figure R2. Comparison of structural alterations of vitamin D-binding protein (GC) *in situ* and *in vitro*. Left: Structural changes of vitamin D-binding protein between healthy and PD CSF are shown. All peptides used for the data analysis are colored in black and the structurally altered peptide is displayed in red. Right: Peptides with significant intensity changes (red) upon 3.3X substrate addition *in vitro* are mapped on the same structure. White indicates peptides that are detected but do not change significantly upon substrate addition. The blue circle shows the *in situ*-changing peptide. The structure is of GC (PDB entry 1J78) with bound vitamin D (yellow spheres).

Second, we tested whether the structural changes in thyroxine-binding globulin that we had observed in PD versus healthy CSF might reflect thyroxine binding. We incubated purified thyroxine-binding globulin with its substrate (L-Thyroxine) in various molar ratios and compared the LiP-MS patterns with and without substrate. In this case, we did not see any evidence for a change in the *in situ*-measured peptide upon substrate addition (data not shown). This may be explained by the fact that an endogenous cleavage event known to influence substrate binding may not have occurred *in vitro*, and we are following this up. We have removed the data on thyroxine-binding globulin from the manuscript to devote more space to the newly added data on proteins that change structurally in both CSF and brain (Figure 4 and Extended Data Figure 4).

Third, we examined the activity of the enzyme BChE in CSF. Our *in situ* data had shown that BChE is structurally altered in the CSF of PD patients relative to healthy individuals, showing significant changes in two structural peptides. We therefore tested in 24 CSF samples whether the activity of this enzyme in CSF differs between healthy individuals and PD patients. We observed no significant difference possibly due to high inter-individual variability in this small set of samples. This may in part reflect the known sensitivity of *in situ* enzyme activity assays to interference from the biological background (e.g., endogenous metabolites or other enzymes that interact with the substrate) and other parameters that are not easy to control, such as pH. This experiment was therefore inconclusive, and we have not added it to the revised manuscript.

Finally, in response to referee 2, we also tested the hypothesis that the bimodal distribution of a structural peptide in FBP-aldolase reflected substrate-bound and –unbound forms of the enzyme but did not see evidence for this. These experiments however did identify another highly variable, unimodally distributed peptide that could reflect the substrate bound state, based on our new *in vitro* data. We have described these *in vitro* experiments in more detail in our response to referee 2 and have included the results in the revised manuscript.

Minor comments:

-On page 3, the authors say the control group had 51 healthy individuals. On page 5, they probably made a mistake and mention 52 healthy individuals. so this should be corrected.

We have 52 healthy donors and are using data from all of them for the variable peptide analysis (Figure 2). However, one of these healthy donors did not have a measurement of total protein content and therefore could not be used for the comparison between healthy and PD (Figures 3 – 5), since this measure is needed for our analytical pipeline. We have made this clearer in the revised text and methods section.

-Figure numbers should be verified as some are repeated, and some figures seem out of the correct order.

-I could not see Figure 5.

We apologize for the figure mislabeling. We have corrected this.

-Why is the Methods section separate from the text, with a separate list of references? Is this a requirement from the journal?

Yes, we are following journal format requirements.

Reviewer #2:

Remarks to the Author:

In the manuscript 'Global analysis of protein structural variation identifies predictors for Parkinson's Disease' Picotti and colleagues present an exciting extension of the scope of their limited proteolysis approach (Lip-MS) to biomarker discovery for Parkinson's disease.

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We thank the reviewer for the positive comments.

Whilst it is understandable that the origin and molecular cause of observed distinct structural features cannot be plausibly explained or investigated in each case, the study as presented to date lacks validation and it is recommended to follow-up on a select few observations using independent methods when revising the manuscript (see also the detailed points below).

We have now added a new analysis of post-mortem brain samples from a small independent cohort and report that several of the proteins that change structurally in PD CSF also do so in PD brain. Further, about half of these are strongly linked to PD, thus strongly supporting their biological- and disease-relevance. Please see our summary at the beginning of this document for a description of these data. The new data are shown in Figure 3F, Figure 4, and Extended Data Figures 3 and 4.

We have also carried out several *in vitro* experiments testing hypotheses about the specific structural changes detected by our global analysis, these are described in more detail in response to the specific points raised below.

Major:

-Healthy and diseased cohorts have marked differences in gender bias. This is sought to be compensated by linear models, but no evidence is provided how well these work. Figure 2 (should be 3) c indicates that the model may overcompensate for sex as low pvalue findings seem underrepresented?

We apologize for the figure mislabeling and have corrected this.

The referee is correct that there is a decrease in small p-values for the sex variable. However, if we examine the p values of the cohort variable when we remove sex, or interactions with sex, or both, from the linear model, there is no substantial change for the number of significant peptides (i.e., 88 peptides for our full linear model, compared to 109 peptides when both sex and interactions with sex were not modeled, 111 peptides when interactions with sex were modeled but not sex, 76 peptides when sex was modeled but not interactions with sex; Figure R3). This indicates that we are taking a conservative approach and minimizing false positive findings. We have now included this plot as Extended Data Figure 3A.

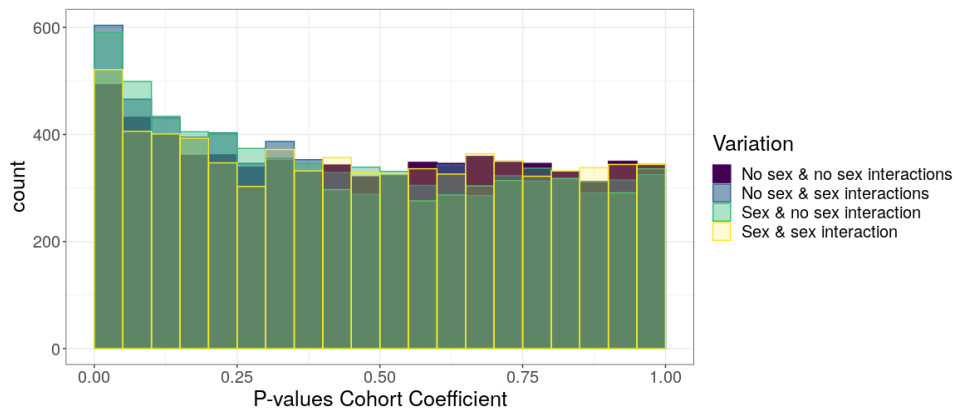


Figure R3. The histogram visualizes the p-values of the cohort variable estimated from the linear model describing effects of structural variation, with the indicated combinations of covariates taken into account. For all models, the first bar (extreme left) indicates significant (<0.05) p-values.

-The bimodality described for the Fructose-bisphosphate aldolase peptide ALQASALK is difficult to understand as the proposed distinct structural states would need to exclusively apply to individuals in the cohorts – e.g. engaged with substrate or not.

The referee is correct that, for proteins like fructose-bisphosphate aldolase with peptides showing bimodal behavior, the data suggest that a given individual would have the protein in almost exclusively one structural state versus another. We assume here that the two well-separated peaks/clouds for LiP peptide intensity indicate two states where the corresponding region of the protein is either more protected (higher peptide intensities) or less protected (lower peptide intensities) from proteolysis, such as one may expect from alternative allosteric or other structural states, or the presence/absence of an interaction partner or specific interaction interface (e.g., due to genetic background differences). We have tried to make this clearer in the text, and have also tested this hypothesis further, as detailed below.

Do other parameters such as sex or batch effects correlate with the observed lip-intensity clusters?

This is an interesting suggestion, and we have now looked into it. For the FBP aldolase bimodal peptide, there is no correlation with batch effects or total protein concentration. In healthy individuals there is no correlation with sex. In PD patients, there is indeed a correlation with sex, with male patients (10/12 patients) in the low-intensity group and female patients (8/11 patients) in the high-intensity group. (Note that we are not able to include all 52 patients in this analysis because the relevant peptide was not detected in all samples). The fact that we did not observe the same phenomenon in healthy donors may be due to lack of statistical power because of the relatively small number of donors. We are following up on these observations but have not further revised the manuscript since this is not the focus of the present study and larger number of patients would be required to address this.

I wonder if simple limited proteolysis experiments with recombinant enzyme in absence or presence of substrate would not be a convenient and convincing means to underpin the finding and its structural interpretation.

Yes, this is in principle a good way to test the hypothesis that the bimodal behavior of the FBP aldolase peptide (ALQASALK) reflects substrate-bound and substrate-unbound states. We have used this approach in other studies (Cappelletti et al, Cell 2021) and have now done this experiment for purified FBP aldolase. While we see structural changes in the *in vitro* enzyme upon substrate addition, these do not map to the *in situ* bimodal peptide (Figure R4). Although the *in vitro* setup may not reproduce cellular events like interactions and chemical modifications, this experiment suggests that the bimodality is likely not caused by substrate binding, and we have revised the text accordingly and added the data to Extended Data Figure 2. Notably however, the *in situ* measured non-bimodal, but highly variable, FBP-aldolase peptide does significantly change upon substrate addition *in vitro*. This could indicate that the interindividual structural variability observed for this site is due to interindividual variability in substrate levels and thus substrate occupancy. We have now mentioned this in the revised manuscript and added the new *in vitro* data to Extended Data Figure 2.

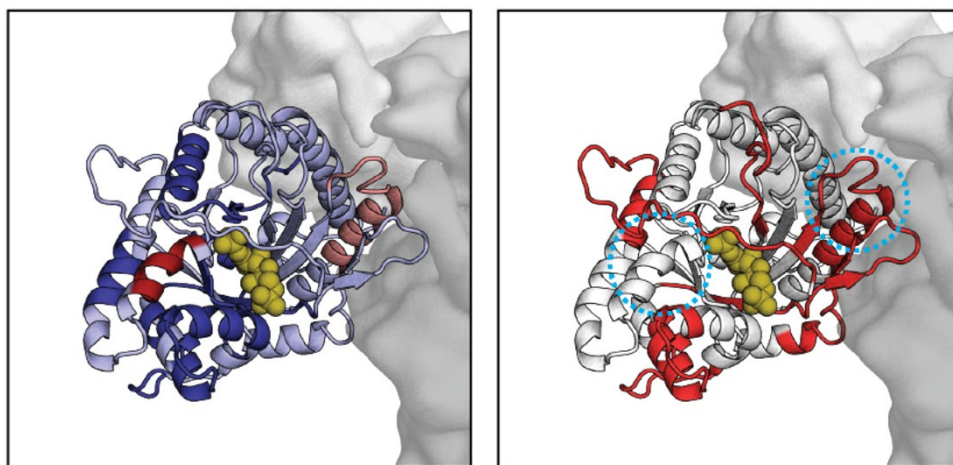


Figure R4. Comparison of structurally variable peptides of FBP-aldolase in situ and in vitro. The left panel shows in situ data and reproduces Figure 2G from the manuscript. The highly variable peptides are shown in dark red (bimodal) and salmon (unimodal). The right panel shows in vitro data; peptides with significant intensity changes upon substrate addition are shown in red. The structure is of human brain FBP aldolase (PDB entry 1XFB), only one subunit of the homotetramer is shown; substrate is represented in yellow. The blue circles indicate the in-situ changing peptides.

This experiment also illustrates some of the challenges of comparing structural proteomics data from purified proteins and complex samples, specifically the importance of mass spectrometric coverage in the ability to interpret the data mechanistically. Further, conclusive validation of LiP-MS-derived structural changes would require high-resolution structural techniques compatible with complex/in situ samples such as CSF and brain, which is currently very challenging if not impossible for some of our target proteins.

-Given the resource character of the manuscript, data descriptions in the supplementary material should be more comprehensive and self-explanatory. For example, the header of supplementary table 5 states: LIP peptide intensities included in the analysis of structural variation. However, there is no column that lists peptide intensities, instead a coefficient and a p-value are listed. It is quite cumbersome to figure out how these were calculated as there is no cross-referencing between supplementary material and materials and methods.

We have now revised the data descriptions of the Supplementary Tables as requested and have more carefully cross-referenced them with the methods. Raw peptide intensities are shown in Supplementary Tables 1 and 2.

-Please avoid the term unbiased in the abstract and elsewhere when referring to LIP-MS, as in any other proteomics method there are several biases in detection related to abundance of the protein of interest and physico-chemical properties of the LIP peptides etc. The approach certainly enables the hypothesis-free and untargeted detection of markers but it is not unbiased.

Good point. We have removed this term.

-An external benchmark how well protein abundance measurements in CSF inform on PD status would be desirable. How well do protein abundance findings in this study correlate with previous studies? Are the structure-based markers fully novel or just distinct from what was found on protein abundance levels in this study? Relevant prior art may include: <https://www.nature.com/articles/s41598-020-59414-4>

We have compared our data with the previous literature. Briefly, we compiled a list of 91 proteins previously reported to change in abundance in CSF or plasma of PD patients from the following publications PMID: 29170290, 31838254, 31190159, 31051162, 30543858, 26907468, 30981640, 31636538, 24691581, 28214264, 31267488) and compared it to the proteins we identify as changing in abundance or in structure both in the CSF and in the brain. Overall, the overlap is low.

For the proteins we have identified as changing in abundance, we see a single previously reported protein in each of our CSF (glial fibrillary acidic protein, GFAP) and brain (complexin-1, CPLX1) datasets. This is consistent with the fact that we overall see few abundance changes between healthy and PD groups (40 proteins in CSF and 27 proteins in brain), possibly because we correct for multiple covariates that could otherwise contribute to abundance changes. We note as well that some of the proteins previously reported to change in abundance were from a single or only a few studies, that previously reported abundance changes for a given protein can be contradictory (e.g., doi.org/10.1371/journal.pone.0257372 detects no GFAP abundance change in CSF of PD patients), and that different studies may report abundance changes in different directions for a given protein.

For the proteins we have identified as structurally changing, the overlap is a little higher. In our CSF data, 3 out of 76 structurally changing proteins have been previously reported to change in abundance in PD; these are apolipoprotein A2 (APOA2), the vitamin D-binding protein (GC), and Neuronal Cell adhesion molecule (NrCAM). In our brain data, 8 out of 334 structurally changing proteins have been previously reported to change in abundance in PD: apolipoprotein E (APOE), apolipoprotein B100 (APOB), neurofilament light polypeptide (NEFL), glutathione-S-transferase-P (GSTP1), pyruvate kinase (PKM), neuromodulin (GAP43), peroxiredoxin-2 (PRDX2), and amyloid beta precursor-like protein 1 (APLP1).

The study mentioned by the referee (Rotunno et al.) is a proteomic analysis of CSF from PD patients in comparison to healthy individuals, reporting abundance changes in 81 proteins in two cohorts with 13 proteins overlapping. There is no overlap between the set of proteins that we and they observe as significantly changing in abundance. Again, this is consistent with the fact that the abundance changes in our cohort are relatively few. The relatively small fold changes (ranging mainly from -0.5 to 0.5) reported by Rotunno et al further corroborate that these effects are quite mild, which may explain the lack of consistency between independent studies. For the proteins for which we report structural changes, 6 in CSF (SGC2, PAM, IGFBP6, NPTX1, TMEM132A, NRCAM) and 13 in brain (NPTX1, C1QB, C1QC, APOD, ENO1, LDHB, OMG, APLP1, CACNA2D1, TPI1, SELENBP1, ALCAM, SPARCL1) were also reported by Rotunno et al. as changing in abundance in the CSF of PD patients.

We have briefly mentioned in the discussion of the revised manuscript that there is some overlap of our detected abundance changes with previous work, and reference the papers, but do not focus on it given that the overlap is so small and given that our study probes structural changes which have not been globally analyzed before.

-A total of 76 proteins were identified as containing differential structural features in PD vs healthy control samples. For a small subset of these interesting discussion is provided how structural changes in these proteins might be linked to mechanisms relevant for disease pathology. However, not in a single case the structural changes were validated through independent means, nor the cause of the observed structural change is identified. E.g. substrate occupancy, dependent variations in PSAT1 should be detectable with other means or on recombinant protein and may inform whether the observed differential LIP-peptides are due to differences in occupancy or may be caused by other mechanisms that may ultimately lead to altered PSAT1 activity.

We have tested hypotheses about the specific structural changes in three proteins we identified as structurally changing in the CSF of PD patients. As for FBP aldolase, described above, this has allowed us to both support and refute specific hypotheses.

First, we used LiP-MS on purified vitamin D-binding protein *in vitro*, to ask whether the single structural change we had observed in PD versus healthy CSF (Figure R2, left) might reflect substrate binding. We incubated purified Vitamin D-binding protein with its substrate (calcitriol) and compared the LiP-MS patterns with and without substrate. The *in situ*-measured structural peptide also changes upon substrate addition *in vitro* (Figure R2, right). Other peptides mapping to the substrate binding site show an even stronger fold change upon substrate addition, but these regions were not well-covered in the *in situ* experiment. These data are therefore consistent with a structural change that occurs upon binding, but further *in situ* validation, including with orthogonal techniques, is needed. We now show these data in Figure 4 and Extended Data Figure 4 and state in the manuscript that further studies are needed.

Second, we tested whether the structural changes in thyroxine-binding globulin that we had observed in PD versus healthy CSF might reflect thyroxine binding. We incubated purified thyroxine-binding globulin with its substrate (L-Thyroxine) in various molar ratios, and compared the LiP-MS patterns with and without substrate. In this case we did not see any evidence for a change in the *in situ*-measured peptide upon substrate addition (data not shown). While this may be explained by the fact that an endogenous cleavage event known to influence the substrate binding may not have occurred *in vitro*, and we are following this up, we have removed the data on thyroxine-binding globulin from the manuscript to devote more space to the newly added data on proteins that change structurally in both CSF and brain (Figure 4 and Extended Data 4).

Third, we examined the activity of the enzyme BChE in CSF. Our *in situ* data had shown that BChE is structurally altered in the CSF of PD patients relative to healthy individuals, showing significant changes in two structural peptides. We therefore tested whether the activity of this enzyme differs between CSF of healthy individuals and PD patients but observed no significant difference possibly due to the high inter-individual variability we observed in the small set of samples we probed (n=24). This may in part reflect the known sensitivity of *in situ* enzyme activity assays to interference from the biological background (e.g., endogenous metabolites or other enzymes that interact with the substrate) and other parameters that are not easy to control, such as pH. This experiment was therefore inconclusive and we have not added it to the revised manuscript.

-Classification characteristics of the models using structural changes are quite promising, my only concern is that the argumentation that this cannot be due to overfitting (lines 425 ff) is vague and not supported by independent data or analyses.

Since we had only a single CSF cohort at hand to build and test models, the ideal option of using a completely independent data set for model testing was not available. Please note that it is particularly difficult to obtain CSF from a relevant number of healthy donors. We therefore used a cross-validation scheme to prevent overfitting. As described in detail in the methods, we subset the data into a training and test group for every single model. Subsequently, we used only the training data to build completely novel linear models to define candidate biomarkers. The results of these linear models were then used to train LASSO models, again exclusively using the training data. Only when evaluating the LASSO models, did we use the test data. Hence, before model evaluation, the test data is never used in any step. We only use the test data to evaluate model performance. We are very aware of the potential issues with such an approach (<https://www.nature.com/articles/s41576-021-00434-9>). Importantly, in our study batch structure was not confounded with the disease status of the donors. Further, we made sure that our normalization approach does not 'leak' information from the training to the test data. We have now tried to clarify this further in the text.

Minor:

-A few more introductory words on the LIP-MS approach would be valuable for the broader readership of this journal. Could be included in the legend of Figure 1 if otherwise less feasible.

We have added this information.

-In times it can be difficult to follow how different experimental constraints led to the numbers of proteins and peptides discussed. A supplementary table detailing, the numbers of proteins and peptides identified in CSF and clarifying how many of these were informative in LIP-MS etc. would be useful.

We have added Supplementary Table 9 detailing this information.

-Figure 2 is labeled as Figure 1 in the manuscript and similarly Figure 2 should be Figure 3 and Figure 5 is labeled as Figure 3. I would recommend carefully checking all references to figures, tables, and other supplementary material.

We apologize for the mislabeling. We have corrected this in the revised version.

-P7, please reference to a supplementary table where all 13 bimodal highly variable peptides are highlighted. Do they behave similar to the fructose-bisphosphate aldolase or rather like semaphorin?

We now highlight all bimodal peptides in Supplementary Table 4 and reference this in the text. They behave similarly to the peptide in FBP-aldolase.

Reviewer #3:

Remarks to the Author: This novel study uses limited proteolysis-mass spectrometry (LiP-MS) for the unbiased detection of global protein structural changes in the CSF of 52 Parkinson's disease (PD) patients in comparison to 51 age-matched healthy controls (HCs). Authors identified 76 proteins as structurally altered, several which have been previously associated with PD (GO enrichment analysis). They were able to disentangle the structural variation from those that occur due to protein abundance variation and showed that the prior is much more significantly associated with the disease status. These structural changes in PD vs. HC were dependent on age but not gender. They also examined the protein structural variability in the CSF of HCs and showed that highly variable peptides had a lower propensity to form beta strands and a higher propensity to form alpha-helices, greater propensity for protein-protein interactions and higher disorder compared to non-variable peptides. This is a useful resource as these highly variable CSF proteins across healthy individuals are unlikely to become good biomarker candidates.

We thank the reviewer for the encouraging comments.

The structurally varied proteins included enrichment of those that have been clearly implicated in the pathogenesis of PD e.g. synaptic proteins, vitamin D, and thus the analysis is mechanistically interesting and valuable. However, it is very difficult to see how this type of expensive and laborious analysis could be taken forward as clinically useful biomarker. It is unlikely that PD CSF samples could routinely be assessed by MS, and there is no information how well this MS analysis could stratify them from other synucleinopathies e.g. multiple system atrophy or even other neurodegenerative disorders e.g. Alzheimer's disease.

The referee brings up several good points and we are currently assembling the cohorts for the mentioned studies.

This manuscript is intended principally to describe the concept of using a global structural proteomic analysis for biomarker discovery and for mechanistic studies of the disease etiology. For the biomarker aspect, much more work is needed before structural signatures (whether the exact ones identified here, or variants, or even quite different ones) can form the basis for clinical tests. The additional studies we plan include validation in independent cohorts, tests of specificity vis-a-vis other neurodegenerative diseases (e.g., MSA, AD, DLB), and tests of structural biomarkers in PD progression.

Assembling the cohorts required for such studies, including for simple independent validation efforts which require CSF from healthy individuals, is a substantial and long-term effort, and in our view is therefore out of the scope and timeline of this discovery study. We have collaborations with three clinical partners (Wilma van de Berg and Charlotte Teunissen at Amsterdam UMC and Bas Bloem at Radboud UMC, Nijmegen) and the cohorts for the above-described studies are currently being assembled. In addition, we are technically optimizing the sensitivity of the LiP-MS analysis to further increase separation between healthy and PD samples.

While the referee correctly cites that mass-spectrometry based CSF measurements can be laborious and expensive in a clinical setting, we point out that well-defined structural signatures could be profiled using targeted proteomic assays (rather than proteome-wide measurements for multiple selected targets) for which there is clinical precedent (PMID: 34136904, PMID: 23396140, PMID: 30487530). Even more clinically viable, however, would be the use of our discovery pipeline as the basis for developing conformational antibodies or antibody panels that detect structural states of interest e.g., that discriminate between PD patients with differential likelihood of developing

dementia. Such antibodies could be developed for ELISAs, for which clinical precedent exists, for instance in diagnosis of Alzheimer's disease.

We recognize that such developments too are many steps down the line. We mention them here to further emphasize that we present this study as an initial demonstration of the potential of global structural analysis to discover a new class of structural biomarker. In response to the referee's criticism, we have made the following changes to the revised manuscript (see also our response to the next point):

(i) We have made more explicit the conceptual nature of our study, and the need for extension to independent cohorts. We have substantially revised the discussion section to make clear that there are many hurdles before our proof-of-principle demonstration of the concept of structural biomarkers could be translated into the clinic, including those raised by the referee.

(ii) We have added discussion on how the structural changes we uncover could be used as the basis for clinical assays based on conformational antibodies, a potential path to clinical use that has precedent in other diseases, but make clear that this too includes unknowns (e.g., defining stable structural states as the basis for developing such tools)

Furthermore, there is no validation of their top structurally varied proteins in an independent cohort, which should be a standard for this type of analysis.

See our response to the previous point. We will indeed conduct an independent validation study, but assembly of the cohort is still in progress. It is thus outside the scope and timeline of this manuscript.

We have however done a global structural analysis of post-mortem brain tissue from a small, independent set of PD patients (n=5) and healthy individuals (n=5). Please see also the summary at the beginning of this document. Briefly, out of the 76 proteins that we initially reported to change structurally in the CSF of PD patients versus healthy individuals, we could measure 27 proteins in the brain data set. Note that the overall overlap between proteins detected in brain and CSF is small (208 proteins), as expected for two quite different sample types. Strikingly, 16/27 of these proteins show a structural change between PD and healthy individuals in brain as well as in CSF. Further, about half of these proteins show strong links to PD based on current literature. Finally, 11 peptides that indicate a structural change between PD and healthy in CSF are also detected in the brain dataset; the majority (8/11) of these peptides change in the same direction between PD and healthy individuals in both brain and CSF. Together, this provides strong support that the structural changes we detect in the CSF reflect changes in the brain, in an independent set of patients, and that the structural signal is informative about disease processes. These new data are shown in Figure 3F, Figure 4, and Extended Data Figures 3 and 4.

It is also disappointing that alpha-synuclein peptides were not detected in the MS analysis due to their low endogenous levels as this obviously would have been very informative information. Authors compared the predictive value of five LiP peptides to oligomeric/total alpha-synuclein ELISA levels that performed together improved the accuracy compared to one marker alone. I think it would have been more interesting to examine the relationship between protein structural changes in low versus high oligomeric/total alpha-synuclein PD groups, as this could reveal something about the possible binding partner changes.

Thank you for the suggestion. We now did this analysis, asking whether any of our 88 putative biomarker peptides correlate with oligomeric/total alpha-synuclein values in PD patients. We used the scaled residuals for this analysis, which reflect how strong the PD effect is in every sample, for each peptide. For most peptides, there is no correlation. Three peptides (one each from calsynenin-3, hemoglobin-alpha and retbindin) show weak but significant correlation between the scaled residuals and the oligomeric/total alpha-synuclein values in PD patients, without FDR correction (Figure R5; hemoglobin-alpha: $\rho = -0.35$, calsynenin-3: $\rho = 0.34$, retbindin: $\rho = -0.38$). We have mentioned this as an observation in the revised text, but do not draw any conclusions from it, given the weak correlation.

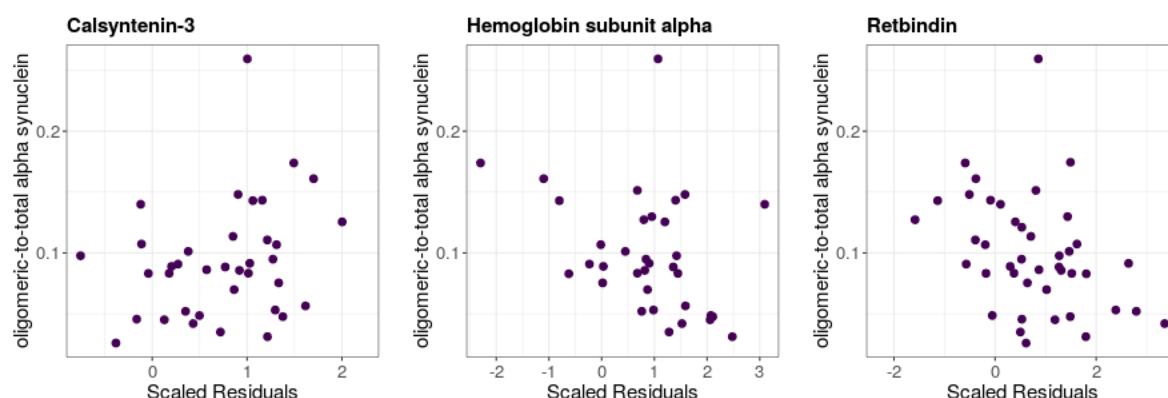


Figure R5. Structural changes in individuals with low versus high oligomer/total aSyn ratios. Scaled residuals for specific peptides from calsyntnenin-3 (left, VNDVNEFAPVFVER), hemoglobin alpha (middle, FLASVSTVLTSK) or retbindin (right, SALGHALPVAAPGAR) are plotted for each individual in the cohort against the measured oligomeric-to-total aSyn ratio. Cohort groups are indicated, each dot represents an individual, each plot shows data for a single peptide.

It is indeed disappointing that aSyn was not detected in CSF with mass spectrometry, but since conformational antibodies for aSyn are available, this protein can already be probed structurally in the CSF. However, aSyn is abundant in the brain and is thus readily detected in our newly added data on brain samples. The data show evidence for a structural change between PD patients and healthy individuals but this does not reach statistical significance due to an outlier individual. Specifically, out of 20 detected aSyn structural peptides, 3 peptides show a significant difference between healthy and PD samples, when the outlier is removed (Figure R1, above); we expect that data on a larger cohort will help resolve this issue. We have mentioned these results in the revised manuscript but do not show the data as we do not see an overall statistically significant change and as the link between aSyn and PD is well established.

I also agree with the authors that this MS analysis should be applied to the brain tissue, where it may be more useful as a research tool to reveal more directly the structural and functional changes related to disease pathology.

We thank the reviewer for this suggestion. As discussed in our responses to earlier points, we have now applied our pipeline to post-mortem brain tissue in a small, independent cohort. Please see our summary of these data at the beginning of this document. We are currently extending these experiments to brain samples from a larger cohort, but this is a separate study.

Minor remarks:

Please include some references to numerous recent studies that have shown alpha-synuclein seeding assays to have a very good sensitivity and specificity for PD patients.

We have added these references.

There are two figures labelled as Figure 1, please correct.

We apologize for the figure mislabeling and have corrected this.

Can you please add any statistical significant differences between the groups to Suppl. Fig 1A? Was the gender different between PD and HC groups?

The age and sex distributions of the PD and healthy groups are shown in Extended Data Figure 1B, and we have added the statistical difference between the groups in Extended Data Figure 1A as requested. There is no statistically significant difference between these groups in terms of age, but there is a difference in terms of sex. This, together with potential interactions of sex with age and other covariates, is precisely why our model includes those covariates, also including interaction terms, to guard against potential bias when defining candidate biomarkers.

Decision Letter, first revision:

Our ref: NSMB-A44796B

17th May 2022

Dear Paola,

Thank you for submitting your revised manuscript "Global analyses of the human structural proteome to identify a new type of disease biomarker" (NSMB-A44796B). It has now been seen by two of the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Structural & Molecular Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

To facilitate our work at this stage, we would appreciate if you could send us the main text as a word file. Please make sure to copy the NSMB account (cc'ed above).

Thank you again for your interest in Nature Structural & Molecular Biology Please do not hesitate to contact me if you have any questions.

Kind regards,
Florian

Florian Ullrich, Ph.D.
Associate Editor
Nature Structural & Molecular Biology
ORCID 0000-0002-1153-2040

Reviewer #1 (Remarks to the Author):

The authors have addressed most issues raised by the reviewers, which is good. However, given the high profile of the journal, dismissing the validation in an independent cohort is not ideal. I would still think this should be done, but I leave it up to the editor to decide. I have no further issues besides this.

Reviewer #2 (Remarks to the Author):

This reviewer appreciates the time and effort that was spent responding to reviewer comments and revising the manuscript. I think the authors did an excellent job and amended the manuscript adequately. It is acknowledged that some of the claims were tuned down by a notch, and focus is

spent on introducing the methodology for discovery of potential biomarkers rather than making strong claims on individual findings. This works well and does neither diminish the relevance of the work nor - in this reviewer's opinion - the relevance for the readership of NSMB. This reviewer supports publication of the amended manuscript.

Decision Letter, final checks:

Our ref: NSMB-A44796B

10th Jun 2022

Dear Dr. Picotti,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Structural & Molecular Biology manuscript, "Global analyses of the human structural proteome to identify a new type of disease biomarker" (NSMB-A44796B). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within two weeks). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: <https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication> for details).

In recognition of the time and expertise our reviewers provide to Nature Structural & Molecular Biology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Global analyses of the human structural proteome to identify a new type of disease biomarker". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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If you have any further questions, please feel free to contact me.

Best regards,

Sophia Frank
Editorial Assistant
Nature Structural & Molecular Biology
nsmb@us.nature.com

On behalf of

Florian Ullrich, Ph.D.
Associate Editor
Nature Structural & Molecular Biology
ORCID 0000-0002-1153-2040

Reviewer #1:

Remarks to the Author:

The authors have addressed most issues raised by the reviewers, which is good. However, given the high profile of the journal, dismissing the validation in an independent cohort is not ideal. I would still think this should be done, but I leave it up to the editor to decide. I have no further issues besides this.

Reviewer #2:

Remarks to the Author:

This reviewer appreciates the time and effort that was spent responding to reviewer comments and revising the manuscript. I think the authors did an excellent job and amended the manuscript adequately. It is acknowledged that some of the claims were tuned down by a notch, and focus is spent on introducing the methodology for discovery of potential biomarkers rather than making strong claims on individual findings. This works well and does neither diminish the relevance of the work nor - in this reviewer's opinion - the relevance for the readership of NSMB. This reviewer supports publication of the amended manuscript.

Final Decision Letter:

18th Aug 2022

Dear Paola,

We are now happy to accept your revised paper "Global analysis of the structural proteome in Parkinson's disease patients to identify a new class of biomarker" for publication as a Article in Nature Structural & Molecular Biology.

Acceptance is conditional on the manuscript's not being published elsewhere and on there being no announcement of this work to the newspapers, magazines, radio or television until the publication date in Nature Structural & Molecular Biology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Structural & Molecular Biology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

After the grant of rights is completed, you will receive a link to your electronic proof via email with a request to make any corrections within 48 hours. If, when you receive your proof, you cannot meet this deadline, please inform us at rjsproduction@springernature.com immediately.

You will not receive your proofs until the publishing agreement has been received through our system.

Due to the importance of these deadlines, we ask that you please let us know now whether you will be difficult to contact over the next month. If this is the case, we ask you provide us with the contact information (email, phone and fax) of someone who will be able to check the proofs on your behalf, and who will be available to address any last-minute problems.

To assist our authors in disseminating their research to the broader community, our SharedIt initiative provides all co-authors with the ability to generate a unique shareable link that will allow anyone (with or without a subscription) to read the published article. Recipients of the link with a subscription will also be able to download and print the PDF.

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Note the policy of the journal on data deposition:
<http://www.nature.com/authors/policies/availability.html>.

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Kind regards,
Florian

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