






A Secretory Vesicle Failure in Parkinson's Disease Occurs in Human Platelets

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Objective: The presence of elevated dopamine (DA) and its major metabolites in the cytosol of neurons has been associated with their vulnerability in Parkinson's disease (PD). Over 99% of the cell's amines are confined to secretory vesicles (SVs), making these structures fundamental in the regulation of cytosolic DA levels. SVs of platelets use similar, if not the same mechanisms to accumulate serotonin in SVs as dopaminergic neurons do to store DA. Hence, any functional defects in platelets probably mirrors events in DA neurons.

Methods: We have isolated fresh platelets from the blood of 75 PD patients, 116 matched controls and 24 patients with Parkinsonism, assaying serotonin handling (basal content, accumulation, secretion and spontaneous leakage).

Results: We found a dramatic decrease in the serotonin content and uptake by SVs, as well as decreased thrombin-induced release by platelets from PD patients but not in those from most Parkinsonism cases. Platelets from PD patients also failed to retain serotonin in SVs.

Interpretation: These findings indicate a functional impairment in the handling of amines by SVs in PD patients. This defect may serve as a biomarker of PD, and the approach described here may be potentially used for the subclinical detection of PD and to establish a platform to assay disease modifying drugs.

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After Alzheimer's disease, Parkinson's disease (PD) is the most common neurodegenerative disorder, and while about 10% of cases have a clear familial origin, most forms of PD are idiopathic (iPD¹). Although the classic description of PD centers on the motor symptoms (dyskinesia, rigidity, tremor), PD also features non-motor symptoms like depression, sleep disorders, anosmia, orthostatic hypotension and digestive ailments. These latter phenomena cannot be explained by the death of nigrostriatal (A9-A10) dopaminergic (DAergic) neurons, indicating a more wide-reaching pathological entity. Normally, patients are

diagnosed with PD once motor problems appear, by when 70–80% of the nigrostriatal DAergic neurons have died. However, subclinical stages of PD may commence long before a diagnosis is reached,² although at present there are no tests that can detect this disease prior to the onset of motor symptoms.

Biological amines, including dopamine (DA), are stored by cells in secretory vesicles (SVs), where they are protected from degradation to reactive oxygen species (ROS). It has been suggested but not yet proven, that defects in the mechanisms leading to DA accumulation in SVs may

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[Correction added on March 26, 2022, after first online publication: Last name and ORCID id have been updated for the author María Dolores Villar-Martinez.]

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produce an excess of cytosolic DA³ and that this would contribute to the pathogenesis of PD. Direct measurement of intravesicular catecholamine concentrations in chromaffin cells have recorded up to 0.8M in mice⁴ or 1M in the cow.⁵ Moreover, measurements from striatal vesicles suggested that approximately 33,000 molecules could be counted per SV, indicating that a 40–50nm diameter vesicle would contain approximately 1M DA.⁶ By contrast, free cytosolic catecholamine concentrations are around 4–50μM,⁷ suggesting that over 99.9% of the cell's amines are stored in SVs and establishing one of the highest known concentration gradients across a biological membrane. SVs are acidic organelles that accumulate H⁺ through a vacuolar ATPase (V-ATPase), which serves to establish a pH gradient (5.5) and a membrane potential (ψ , +80mV) that help membrane carriers (VMAT-2, VNUT) transport amines or Ca²⁺. Each biological amine (DA, noradrenaline, adrenaline, serotonin and histamine) accumulates in the SVs through a similar, if not the same transport systems in distinct secretory cells and neurons.^{8, 9}

Platelets represent the main reservoir of serotonin (>99%) in the blood, where it is stored in dense granules. Platelets do not synthesize serotonin but rather, they take it up from the plasma via the serotonin transporter (SERT) following its release by gut enterochromaffin cells, although serotonin can also cross the plasma membrane by diffusion. Isolated platelets provide a model to directly study the activity of SVs, as they take-up serotonin in a concentration-dependent manner and they release it by exocytosis in response to secretagogues like thrombin.¹⁰ We hypothesized that if the chronic toxic effect of cytosolic DA could provoke PD, the main source of DA might be SVs and that the SVs of other cells that store amines might be also affected, such as those in platelets. Therefore, we studied whether the storage and handling of amines by SVs in platelets is disrupted in PD. Indeed, we demonstrate significant alterations to the handling of serotonin by SVs in PD patients that involves: (i) limited content, (ii) reduced uptake, (iii) less release after thrombin stimulation, and (iv) higher leakage from SVs. As a result, we propose that the deregulation of SVs, and an associated increase in free monoamines and Ca²⁺ may occur during the pathogenesis of PD. Moreover, we suggest that identifying this defect could offer a means to achieve an early diagnosis of PD and a means to establish screening programs. In addition, this model could constitute a very valuable tool for the testing of disease modifying drugs.

Methods

A detailed description of the methods employed here is given in the Supplemental Material.

Patients

This study was approved by the Ethical Committee at the University of La Laguna (CEIBA2017-0244) and by the Ethical Committee at the Canary Islands Health Services CHUC_2020_80). Blood samples were obtained by venipuncture and identified using anonymized codes. All personal information related to patients and controls were maintained by the neurologists at the Neurology Services that participated in these studies (MDV, AA, MP, JNL and FC). The analytical team (PM and RB) performed their analysis blind to the source of the samples, receiving only the blood samples and codes. Only after the study was completed were the individuals classified as PD, Parkinsonism or controls. Patients were diagnosed at the Neurology departments of the University Hospital of Canarias (HUC) and *Nuestra Señora de la Candelaria* (HUNSC) based on clinical criteria and DaTSCAN.

Platelet Purification

A detailed description of the procedure to obtain the platelets is given in the Supplemental material. Briefly, two blood samples of 9mL were obtained by venipuncture (BD-Vacutainer[®], with 18.0mg K₂-EDTA; Plymouth, UK) and the platelets were purified by sequential centrifugation at room temperature. Platelets were maintained in a humidified atmosphere at room temperature by gentle rotation, and the assays were performed in 96-well plates with conical bases and hydrophobic surfaces. Platelets were then plated at a density of 10⁷/well and they were used within 24h of isolation.

Analysis of Serotonin

We used an isocratic UPLC system coupled to electrochemical detection to analyze serotonin, with isoproterenol as an internal standard. Chromatograms were recorded using tailor-made routines written in the LabView[®] 2011 platform (National Instruments, Austin, TX, USA) for a Macintosh computer. The analysis was carried out using locally written macros for Igor Pro (Wavemetrics, Lake Oswego, OR, USA).

Protein Analysis

To normalize serotonin measurements, we quantified the total protein using the bicinchoninic acid method according to the manufacturer's guidelines (Sigma-Aldrich) and using bovine serum albumin for calibration.

Results

Impaired Serotonin Uptake in Platelets From PD Patients

We detected a much lower basal serotonin content in platelets isolated from PD patients than in those from the

matched non-PD control individuals (Fig 1). As the serotonin naturally stored by platelets is taken up from the blood, this analysis directly reflects the capacity of SVs to accumulate serotonin when platelets are exposed to normal serum concentrations of this amine. The lower basal serotonin content was not due to deficient serotonin

release from enterochromaffin cells, as impaired serotonin uptake was evident over a wide range of concentrations tested (3nM–30µM: see Fig 1B). Incubation with higher concentrations of serotonin (100–1,000µM) produced a non-saturable profile of non-specific uptake (Fig S1a, SM). The average basal serotonin content of PD platelets was 1.46 ± 0.13 nmol/mg protein ($n = 75$, mean \pm SEM) as opposed to 3.03 ± 0.13 nmol/mg protein in those isolated from the matched controls ($n = 116$). Based on the uptake curves (see Fig 1B), we exposed platelets to a serotonin concentration of 10µM, close to that producing maximal uptake and that was therefore least influenced by non-specific uptake (see below). At this concentration (10µM) significantly less serotonin was taken up by platelets from PD patients (7.07 ± 0.31 nmol/mg protein) than by platelets from the matched controls (11.70 ± 0.36 nmol/mg protein).

Based on the serotonin content detected (see Fig 1A), we established arbitrary 'pathological' serotonin thresholds (numbers in bold) for the basal content (1.71nmol/mg protein, the lowest SD of the control values) and for the relative accumulation after a 2h incubation with serotonin (10µM, 7.87nmol/mg). Low serotonin levels and uptake were observed in platelets from most PD patients, irrespective of whether they were receiving L-DOPA treatment (see Fig 1D). Therefore, PD patients at earlier stages of the disease, prior to L-DOPA therapy, showed the same changes in serotonin as

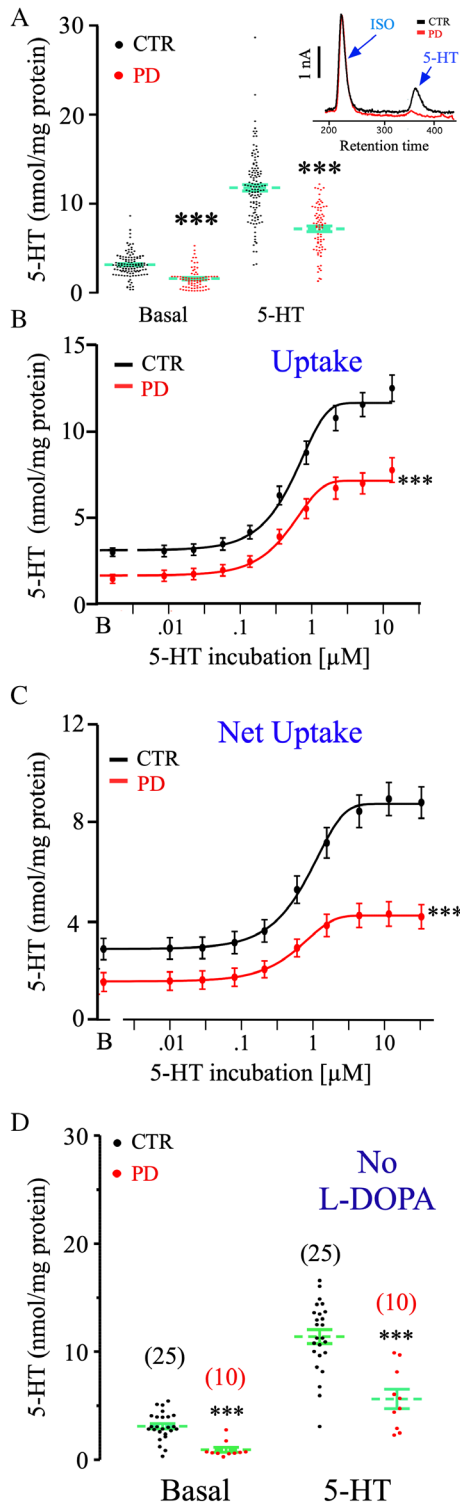


FIGURE 1: (legend on next column.)

FIGURE 1: Secretory granules from the platelets of PD patients have less naïve serotonin and severely reduced serotonin uptake. Platelets were isolated using our optimized protocol to measure serotonin. **A.** The pooled data from 75 PD patients (red dots) and 116 controls (CTR, black dots), representing the average of duplicate measurements normalized to the total protein. The panel shows the basal serotonin content and its uptake (5-HT) after 2h incubation with serotonin (10µM). The horizontal green dashed lines indicate the mean and the solid horizontal lines the standard errors: *** $p < 0.001$ (Mann-Whitney's test). Inset. typical chromatograms from controls and PD patients to quantify serotonin by HPLC with electrochemical detection using isoproterenol (200nM) as an internal standard. **B.** The serotonin uptake is reduced in PD patients in the range of 3nM to 30µM ($n = 59$ PD and $n = 61$ Controls). **C.** Serotonin uptake after the subtraction of the non-specific component (1µM reserpine, $n = 39$ PD and $n = 35$ controls; Fig 1b SM): means \pm 95% confidence interval; *** $p < 0.001$ (ANOVA). **D.** Pooled data (means \pm SEM) from 10 untreated PD patients (red) and 25 random controls (CTR, black), representing the average of the duplicates normalized to the total protein. The horizontal green dashed lines indicate the mean and the solid horizontal lines the standard errors. The panel shows the basal serotonin content and its uptake (5-HT) after 2h incubation with serotonin (10µM): *** $p < 0.001$ (Mann-Whitney's test).

those patients at later stages of the disease. No significant differences were found in the distinct age segments or for gender (Table S5, SM).

We also assayed the serotonin turnover in platelets from patients diagnosed with Parkinsonism disorders (iatrogenic, multiple system atrophy, Lewy body dementia, progressive supranuclear palsy, vascular origin). Although there are few subjects with each of these disorders, most patients with Parkinsonism had normal serotonin values, such that these conditions probably had a distinct origin to that of iPD (Table S1, SM).

Treatment with the anti-thrombotic drug acetylsalicylic acid did not seem to affect SV function (Fig S3, SM). Platelets were unable to transform L-DOPA to DA or 5-hydroxytryptophan to serotonin, indicating that they lack a functional L-aromatic amino acid decarboxylase that transforms these neurotransmitter precursors into their corresponding amines (Fig S4, SM). Not all clinically diagnosed PD patients had ‘*pathological*’ serotonin platelet values (Table S2, SM) and we consider that these patients may reflect different forms of PD, or that these differences were due to the state of platelets at the time of blood donation, misdiagnosis or other reasons that are currently being examined. Concomitant diseases seem to affect our data, such as: thrombocytopenia, which causes the overestimation of serotonin; hyperthyroidism, which affects platelet protein content and function; and perhaps dyslipemias, which increase non-specific uptake. A future goal is to refine our analyses by expressing the data relative to the total platelet mass (cell count \times platelet volume). Nevertheless, there were three cases with pathological DaTSCAN images (076L, 839N and 568T) in which serotonin content and uptake was normal, which corresponded to approximately 4% of our PD patients. At present we cannot offer a clear explanation of these results. The current Covid-19 pandemic has meant these patients cannot be re-evaluated clinically at this time, although these ‘*outliers*’ may constitute another form of PD that does not involve SV defects.

Non-Specific Uptake

The laboratory handling of serotonin is not straightforward as it binds non-specifically to plasticware. Moreover, platelets are cell fragments with a large membrane to volume ratio, particularly due to their small size, irregular shape and the presence of a tubular system. Serotonin is stored at very high concentrations in dense (δ -) granules but there are only 4–8 δ -granules per platelet. Since serotonin is a lipophilic moiety that binds to cell membranes, the relative importance of non-specific uptake must be evaluated. Non-specific uptake can be estimated indirectly using drugs that block the activity of SV transporters:

bafilomycin A1 that irreversibly blocks V-ATPase; and reserpine or tetrabenazine that irreversibly and reversibly block VMAT-2, respectively. Reserpine and tetrabenazine had a similar effect on serotonin uptake, while bafilomycin had a slightly less potent effect. Furthermore, the component resistant to the VMAT-2 blockers was not significantly different in the PD (2.44 ± 0.28 nmol/mg protein) and matched control platelets (2.49 ± 0.26 nmol/mg protein), indicating that this is due to the non-specific serotonin binding (Fig S2, SM).

To rule out any contribution of non-specific uptake, we incubated the platelets with reserpine and no differences in the concentration-uptake curves were evident between 0.1–10 μ M reserpine (Fig S1b, SM). To calculate the specific uptake, we studied a group of controls ($n = 35$) and PD patients ($n = 39$) in the presence and absence of reserpine (1 μ M), calculating the specific uptake by subtracting the reserpine resistant component from the total uptake (see Fig 1C). Even considering the smaller number of individuals studied, the differences in serotonin uptake (10 μ M/2h) between controls (9.00 ± 0.34 nmol/mg protein) and PD patients (4.29 ± 0.25 nmol/mg protein) were even more evident, reflecting the impairment of SVs in PD platelets (see Fig 1C; Fig S6, SM). As a result, we established the ‘*pathological*’ threshold of specific serotonin accumulation at 7.02 nmol/mg protein (the lowest SD of the control values), as reflected in the tables.

Detecting Subclinical Cases

One of the goals of this study was to explore the possibility that subclinical PD can be detected prior to the appearance of motor symptoms. We identified five matched controls with abnormally low serotonin levels (Table S3, SM; Fig S5, SM), yet at this time it is unclear whether they may eventually develop PD. Several clinical situations have been associated with reduced basal serotonin in platelets, such as an association with major endogenous depression.¹¹ When the ability of SVs to concentrate amines was evaluated, the serotonin uptake by the platelets from three individuals diagnosed with major depression was ‘*normal*’. One patient (213J) initially considered as a possible case of PD was later diagnosed with major depression, consistent with the serotonin data as SV uptake was normal even though the basal serotonin levels were low (Table S3, SM; Fig S5, SM).

Serotonin Release

Platelets release serotonin by exocytosis when incubated with a physiological secretagogue like thrombin. Thrombin evokes a rapid secretory response that usually lasts less than 20s and hence, serotonin release in response to 4U/mL of thrombin can be characterized. As expected,

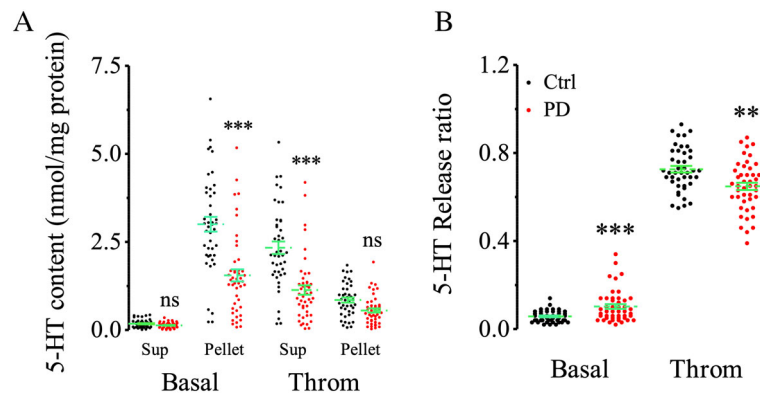


FIGURE 2: Secretory mechanisms are impaired in platelets from PD patients. A. Serotonin was quantified in the supernatant (Sup) and the platelet pellet was recovered by centrifugation (Pellet = serotonin not secreted). Platelets from healthy individuals (CTR) and from PD patients (PD) were stimulated for 90s with thrombin (4U/mL): ns, not significant; *** $p < 0.001$ (ANOVA). B. The fractional release (=serotonin release/(serotonin released + serotonin remaining)) indicated there was more serotonin leakage from PD platelets but less thrombin-evoked release ($n = 43$ Control, $n = 46$ PD): ns, not significant; ** $p < 0.01$; *** $p < 0.001$ (unpaired Student's t test).

platelets from PD patients release significantly less serotonin (approximately 50%) than platelets from matched controls (Fig 2A). Interestingly, SVs from PD platelets leak significantly more serotonin in the absence of stimuli (basal conditions) than those from controls. Therefore, these data indicate that in SVs from PD patients the mechanisms to retain the stored amines are also impaired (see Fig 2B).

Discussion

The presence of higher cytosolic DA levels has been proposed to be a major toxic insult that causes neurodegeneration of nigro-striatal neurons. As SVs are the main amine reservoir in both neurons and platelets, which seem to share the basic mechanisms of amine accumulation, we explored the handling of serotonin by platelets from PD patients. Currently it is not possible to measure free cytosolic serotonin in platelets. However, when compared to matched controls, whole platelets from PD patients had: (i) a significantly lower basal serotonin content, (ii) a highly reduced ability to take up serotonin, (iii) a reduced secretory response to a secretagogue, and (iv) increased spontaneous serotonin leakage. These differences were not observed in patients with parkinsonism and indeed, these serotonin tests are currently used by our neurologists as a differential diagnostic tool of parkinsonism and PD. However, due to the limited number of subjects diagnosed with specific parkinsonism syndromes ($n = 24$), this differential diagnostic tool should be used with caution. We used serotonin rather than DA because it is a natural component of platelets and its uptake is not affected by L-DOPA treatment (see Fig 1), unlike DA.^{12, 13}

This is not the first time platelets have been related to PD. Decreased basal serotonin in conjunction with normal uptake was reported some years ago¹² and a reduced ability for DA uptake by SVs was noted in platelets from untreated PD although these values apparently return to normal after L-DOPA treatment.¹³ These results were probably affected by the small contribution of d-granules to the total granule content. A reduction in the ¹⁴C-DA uptake has been found, yet some of the pioneering findings of this group were surprisingly largely ignored.¹⁴ Significantly low plasma serotonin levels have been associated with non-motor symptoms,¹⁵ although elsewhere no differences in basal serotonin content were detected.¹⁶ Similarly, no alterations in serotonin uptake by platelets were found in an earlier study, although this may have been due to the methodology followed,¹⁷ and to the best of our knowledge, no functional studies have been carried out on human platelets from PD patients.

Although other amines like noradrenaline (NA) and serotonin may also be converted into noxious species, DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAL) are especially neurotoxic due to the generation of ROS^{18, 19} and the capacity of DOPAL to also induce α -synuclein aggregation.²⁰ The principal physiological way to overcome these metabolic changes is by rapidly confining DA in SVs, where it is protected from degradation, and hence, functional defects in SVs may produce excess cytosolic DA (for a review of DA turnover see Ref. 3). SVs also represent an important Ca^{2+} reservoir,²¹ and the combination of high cytosolic DA and Ca^{2+} may act synergistically to promote neurotoxicity.²² Moreover, dysfunctional NA and serotonin accumulation in SVs could explain the non-motor symptoms of PD, such as sleep disorders,²³ major depression,¹¹ digestive disorders,²⁴ as

well peripheral signs like orthostatic hypotension and bradycardia.

Multiple mechanisms may drive the functional impairment of SVs, involving V-ATPase, VMAT-2, VNUT or other proteins/factors, and PD could reflect any or a combination of these. However, the only one considered to date is that involving VMAT-2, which was notably reduced in post-mortem striatal SVs from dopaminergic neurons.²⁵ In addition, the significant spontaneous leakage of serotonin from PD platelets (see Fig 2B) suggests poor amine retention by SVs.

It is not clear whether our findings lie at the origin of the disease or if they are a mere consequence of PD, yet our results support the former as it would seem unlikely that the impaired serotonin uptake by platelets were a result of the evolution of PD. Our data also indicate that this alteration in amine handling is not caused by the treatment of PD as it was also evident in untreated patients. The neurodegeneration associated with PD occurs in the decades prior to the onset of the first symptoms but there are still no diagnostic tools capable of reliably detecting the disease in these prolonged subclinical phases. Although several biomarkers have been proposed for the early diagnosis of this disease, including α -synuclein,²⁶ or DJ-1 deglycase or β -glucocerebrosidase activity in cerebrospinal fluid (CSF²⁷), their cost, inconvenience and reliability make them unsuited to be used for subclinical diagnosis. The serotonin testing described here may be complementary to examining genetic features of PD, such as LRRK2 (leucine-rich repeat kinase 2²⁸), PINK1 (PTEN-induced putative kinase 1) or PRKN (Parkin RBR E3 Ubiquitin Protein Ligase²⁹) involvement, as most of the monogenic causes are incompletely penetrant and positive genetic testing in asymptomatic individuals does not provide sufficient diagnostic evidence of subclinical PD. By contrast, this functional study of SVs in platelets suggests that such an approach might be capable of detecting PD in a large proportion of patients (approximately 77%) and perhaps even in more patients (approximately 91%) if the non-specific component is removed.

As serotonin uptake is the result of more factors, it was not our aim to propose that the events described here are due to the malfunction of any specific protein. We propose the serotonin test can be used for PD diagnosis and to achieve a differential diagnosis from Parkinsonism. Given that PD neurodegeneration begins decades before the appearance of motor symptoms and that SV failure may occur years before the onset of the first symptoms, serotonin assays might be able to detect the disease decades before the onset of its first symptoms. Serotonin testing is cheap, rapid, and reliable,

and it can be implemented in any clinical laboratory. Moreover, the functional analysis of serotonin turnover also constitutes a very promising tool for the discovery of disease modifying drugs that can be carried out directly on patient's cells.

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Author Contributions

RB, MDV and PM contributed to conception and design of the study. AA, MP, MD, JNL, FC, RB and PM contributed to the acquisition and analysis of data. RB, MDV and PM contribute to drafting the text and preparing the tables and figures.

Potential Conflicts of Interest

Authors declare no competing financial or non-financial interests.

Data Availability

All the data generated or analysed in this study are included in the article and its supplementary information files. Any additional information will be available from the corresponding author on reasonable request.

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