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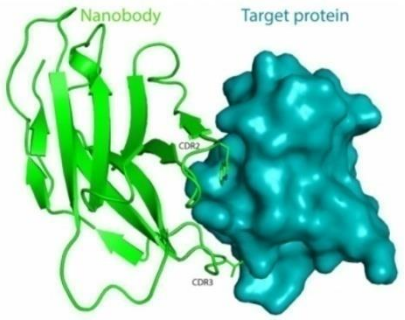
Master of Science

"Molecular and Applied Physiology"

Application of novel Nanobodies to already existing LRRK2 biomarker assays



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Περίληψη

Η νόσος του Πάρκινσον (ΝΠ) είναι η δεύτερη πιο διαδεδομένη νευροεκφυλιστική διαταραχή που εκδηλώνει προοδευτική κινητική δυσλειτουργία αλλά και μη-κινητικά συμπτώματα. Η LRRK2 είναι ένα διπλό ένζυμο-κινάση/GTPάση η οποία υφίσταται ως μονομερής ή διμερής, με συμμετοχή τόσο στην ιδιοπαθή όσο και στην οικογενή ΝΠ. Η G2019S μετάλλαξη της πρωτεΐνης φαίνεται να είναι η πιο συχνή σημειακή μετάλλαξη και κυρίως συσχετίζεται με αυξημένη ενεργότητα κινάσης και προκαλεί την νέκρωση βασικών νευρώνων. Είναι γνωστό ότι η ενεργότητα της κινάσης είναι συγκεντρωμένη σε διμερείς μορφές και στην περίπτωση της μεταλλαγμένης LRRK2, το ομοδιμερές παρουσιάζει την μεγαλύτερη ενεργότητα. Κατά τη διάρκεια προηγούμενης έρευνας βιοδεικτών, αποδείξαμε ότι οι φορείς της μετάλλαξης G2019S έχουν αυξημένη ενεργότητα κινάσης σε μονοπύρηνα κύτταρα περιφερικού αίματος (PBMCs). Παρόλα αυτά, η ακριβής διαμόρφωση της LRRK2 που προκαλεί την εκδήλωση της ΝΠ και την εξέλιξή της δεν είναι ακόμη ξεκάθαρη. Προκειμένου να αντιμετωπίσουμε αυτό το ζήτημα, χρησιμοποιήσαμε τα καινοτόμα νανοσώματα (Nbs) - θραύσματα αντισωμάτων αποκλειστικά βαριάς αλυσίδας προερχόμενα από καμηλίδες, που εμφανίζουν διαφορετική συγγένεια ανάλογα με την διαμόρφωση του στόχου τους. Συνεπώς, χρησιμοποιώντας τα Nbs για ποικίλα είδη της LRRK2, ελέγξαμε για τη συγγένειά τους με διμερείς μορφές της και χρησιμοποιήσαμε επιλεγμένους κλώνους για μετρήσεις κλινικών δειγμάτων. Τα δείγματα που χρησιμοποιήσαμε προέρχονταν από την ίδια ομάδα με την προηγούμενη μελέτη βιοδεικτών, επιτρέποντάς μας να αποκτήσουμε περισσότερες πληροφορίες σχετικά με το προφίλ των ασθενών με ΝΠ και να εξερευνήσουμε τη χρήση των Nbs ως εργαλεία για μελέτη βιοδεικτών.

Abstract

Parkinson's Disease (PD) is the second most common neurodegenerative disorder, manifesting progressive motor dysfunction as well as non-motor symptoms. Leucine-rich repeat kinase 2 (LRRK2) is a kinase/GTPase dual enzyme that exists as a monomer or a dimer, with implications in both idiopathic and familial PD. The G2019S mutation of the protein appears to be the most frequent point mutation and mainly correlates with increased kinase activity and causes the death of primary neurons. It is known that the kinase activity is concentrated in the dimeric species and in the case of mutant LRRK2, it is the homo-dimer which posseses the greatest activity. During a previous biomarker study, we showed that G2019S mutation carriers have hyperactive kinase activity in peripheral blood mononuclear cells (PBMCs), however the specific LRRK2 conformation state for PD manifestation and during the disease progression is still unclear. In order to tackle this issue, we are now using the novel nanobodies (Nbs), camelid-derived, heavy-chain only antibody fragments that show differential affinity depending on their target's conformation. Therefore, by using Nbs for various LRRK2 species, we screened for their affinity for LRRK2 dimers and applied selected clones for clinical sample measurements. The samples used were from the same cohort of our previous biomarker study, allowing us to gain further information about these groups' PD profile and explore the use of Nbs as biomarker tools.

Keywords: Parkinson's Disease, LRRK2, G2019S, Biomarkers, Nanobodies, PBMCs

Table of contents

Abbreviations

PD: Parkinson's Disease

SNpc: substantia nigra pars compacta

iPD: idiopathic Parkinson's Disease

ROS: reactive oxygen species

LB: Lewy Bodies

MSA: Multiple System Atrophy

LD: levodopa

DA: Dopamine

MAO-B: monoaminooxidase B

LRRK2: Leucine-rich repeat kinase 2

DAPK1: Death-associated protein kinase 1

MASL1: Malignant fibrous histiocytoma amplified sequence 1

LRRK 1: Leucine-rich repeat kinase 1

ROC: Ras of complex proteins

COR: C-terminal of ROC

PBMC: peripheral blood mononuclear cells

LRR: Leucine-rich repeat

ERM: Ezrin, Radixin and Moesin

Rab: Ras-related protein in brain

LPS: lipopolysaccharide

GEF: guanine nucleotide exchange factor

GAD: G-proteins activated by nucleotide dependent dimerization

DLS: dynamic light scattering

BN PAGE: Native polyacrylamide gel electrophoresis

EM: electron microscopy

PTM: post-translational modification

HEK: human embryonic kidney

IgG: Immunoglobulin G

Ab: antibody

Fab: fragment antigen domain

Fc: fragment crystallizable

CDR: complementary-determining region

Nb: nanobody

PCR: polymerace chain reaction

ELISA: enzyme-linked immunosorbent assay

STED: stimulated emission depletion

STORM: stochastic optical reconstruction microscopy

PALM: photoactivated localization microscopy

GFP: green fluorescent protein

RFP: red fluorescent protein

F3H: fluorescence three-hybrid assay

LP: localization protein

FRET: Förster resonance energy transfer

AP-MS: affinity-purification mass spectrometry

NMR: nuclear magnetic resonance

BBB: blood-brain barrier

GFAP: glial fibrillary acetic protein

SrtA: Sortase A

SA: streptavidin

AP: acceptor peptide

FTIR: Fourier-transformed infrared spectroscopy

1. Introduction

1.1. Parkinson's Disease

Since the first in depth description of "shaking palsy" by James Parkinson in 1817 [1], knowledge and better understanding of Parkinson's Disease has come a long way. In fact, beyond its initial perception as a movement disorder, it has become clear that Parkinson's Disease (PD) is a neurodegenerative disorder that also involves non motor symptoms that may arise, many times preceding the onset of motor symptoms. It is the most common movement disorder and the second most common neurodegenerative disease. PD's highest risk factor is aging and it is estimated that around 1% of people over 60 years are manifesting the disease [2].

PD is characterized by tremor, rigidity and bradykinesia with the possible addition of postural instability. These symptoms are predominately occurring due to the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Nevertheless, there can be other non motor symptoms as hyposmia, rapid eye movement sleep behavior disorder, depression or constipation that can either precede or follow the motor symptoms [3]. In addition, cognitive deficits can be present especially at the later stages of PD development.

The majority of PD cases are sporadic (idiopathic PD, iPD), but there is approximately 10% of the total cases that report a family history. However, even among the iPD cases about 5% of those are gene associated [3]. Genes involved in monogenic forms of PD are SNCA (α-synuclein) and LRRK2 responsible for autosomal dominant PD and PINK1, PARK7 (DJ-1), PARK2 (Parkin) and ATPase 13A2 [4] among others shown below (*table 1*). Also, GBA (glucoserebrosidase) gene mutations while mainly associated with Gaucher's Disease, are a genetic risk factor for PD [5]. In addition to genetic causes, environmental contributors, especially pesticides like dieldrin, rotenone and paraquat are identified as high risk factors of PD. Excess iron in the brain can also be neurotoxic and is linked to PD by causing reactive oxygen species (ROS) [6].

Symbol	Gene locus	Disorder	Inheritance	Gene	Status and remarks	Mode of identification
PARK1	4q21-22	EOPD	AD	*SNCA*	Confirmed	Linkage analysis
PARK2	6q25.2–q27	EOPD	AR	*Parkin*	Confirmed	Linkage analysis
PARK3	2p13	Classical PD	AD	Unknown	Unconfirmed; may represent a risk factor; gene not found since first described in 1998	Linkage analysis
PARK4	4q21–q23	EOPD	AD	*SNCA*	Erroneous locus (identical to *PARK1*)	Linkage analysis
PARK5	4p13	Classical PD	AD	*UCHL1*	Unconfirmed (not replicated since described in 1998)	Functional candidate gene approach
PARK6	1p35–p36	EOPD	AR	*PINK1*	Confirmed	Linkage analysis
PARK7	1p36	EOPD	AR	*DJ-1*	Confirmed	Linkage analysis
PARK8	12q12	Classical PD	AD	*LRRK2*	Confirmed; variations in *LRRK2* gene include risk-conferring variants and disease-causing mutations	Linkage analysis
PARK9	1p36	Kufor-Rakeb syndrome; atypical PD with dementia, spasticity, and supranuclear gaze palsy	AR	*ATP13A2*	Confirmed; but complex phenotype that would not be mistaken for early-onset or classical parkinsonism	Linkage analysis
PARK10	1p32	Classical PD	Risk factor	Unknown	Confirmed susceptibility locus; gene unknown since first described in 2002	Linkage analysis
PARK11	2q36-27	Late-onset PD	AD	Unknown; not *GIGYF2*	Not independently confirmed; possibly represents a risk factor; gene not found since first described in 2002	Linkage analysis
PARK12	Xq21–q25	Classical PD	Risk factor	Unknown	Confirmed susceptibility locus; possibly represents a risk factor; gene not found since first described in 2003	Linkage analysis
PARK13	2p12	Classical PD	AD or risk factor	*HTRA2*	Unconfirmed	Candidate gene approach
PARK14	22q13.1	Early-onset dystonia-parkinsonism	AR	*PLA2G6*	Confirmed	Linkage analysis (homozygosity mapping)
PARK15	22q12–q13	Early-onset parkinsonian-pyramidal syndrome	AR	*FBX07*	Confirmed	Linkage analysis
PARK16	1q32	Classical PD	Risk factor	Unknown	Confirmed susceptibility locus	Genome-wide association studies
PARK17	16q11.2	Classical PD	AD	*VPS35*	Confirmed	Exome sequencing
PARK18	3q27.1	Classical PD	AD	*EIF4G1*	Unconfirmed; recently published (Chartier-Harlin et al. 2011)	Linkage analysis

AD, autosomal dominant; AR, autosomal recessive.

Table 1: Genes associated with PD [3].

The main pathological feature of PD is the loss of dopaminergic neurons in SNpc and mostly neurons that project to the dorsal putamen of the striatum. Other brain regions are also affected though, including locus ceruleus, nucleus basalis of Meynert, amygdala and hypothalamus [7]. However, the dopaminergic neuronal system is not the only one that gets involved, as glutamatergic, cholinergic tryptaminergic, noradrenergic, adrenergic, serotoninergic and peptidergic systems can also be affected [4]. Along with the dopaminergic neuronal loss, the presence of cytoplasmic inclusions called Lewy Bodies (LB) was established as the hallmark and diagnostic criterion of PD.

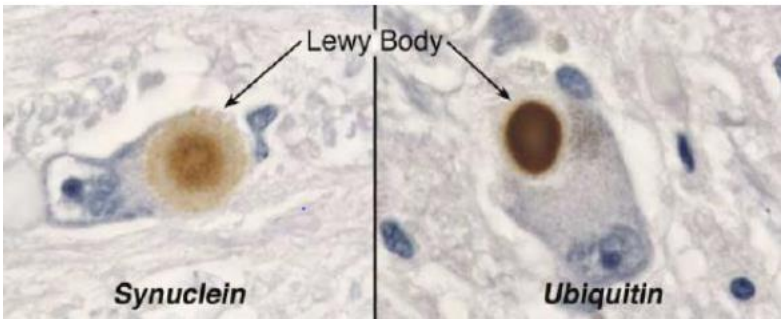

Figure 1: Immunohistochemical labeling of Lewy Bodiesin a SNpc dopaminergic neuron, stained against α-synuclein and ubiquitin [8].

Other than in PD, LB-like inclusions are involved in Lewy Body Dementia [9] and Multiple System Atrophy (MSA) [10] and they mostly consist of α-synuclein and ubiquitin. α-synuclein is a 140 amino acid presynaptic protein. It is noted that variants, mutations and gene duplications or triplications are high risk factor of PD and are present in the majority of PD cases. Lysosomal and proteasomal degradation pathways are suspected as a mechanism in the pathogenesis of PD and there is also evidence of mitochondrial dysfunction [4].

There is no cure in PD and all the current treatment methods focus on the motor aspect of the disease, targeting the symptoms. Levodopa (LD) is the most common medication since its introduction in the 1960s. It's a precursor of dopamine that crosses the blood brain barrier and coverts to dopamine by the CNS or the periphery [11]. However, after some time, it is observed to lead to motor complications especially for the early-onset PD patients [12]. Apart from LD, dopamine (DA) agonists act directly on dopamine receptors by mimicking the endogenous neurotransmitter, or stimulating its release. DA offers an alternative for younger PD patients with mild to moderate symptoms because they reduce the LD related long term problems [13]. The use of DA agonists though is not suggested because of serious side effects. A different approach is with monoaminooxidase B(MAO-B) inhibition stabilizing dopamine levels in the synapses. However, at higher levels, MAO-B inhibitors lose selectivity and inhibit MAO-A as well, with the risk of other complications as hypertension [14].

The lack of a dependable drug linked to the need for reliable biomarkers. According to NIH a biomarker is "*a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*". An optimal biomarker for PD should differentiate it from similar disorders, diagnose early symptoms or mechanisms and prove reliable and reproducible. Variations in the *SNCA* gene, which cause PD and α-synuclein aggregations, post-translational protein modifications and various protein conformations show a strong association with PD and can be detected or measured in biofluids and peripheral tissues [15].

As mentioned, LRRK2 is a major contributing factor of the disease initiation and progression, being involved in both familial and sporadic forms of PD. In this project we are focusing on its conformation and its utility as potential biomarker.

1.2. LRRK2

In a genome-wide linkage analysis in 2002, PARK8 locus was mapped in a Japanese family with autosomal dominant parkinsonism with similar clinical features with sporadic PD [16]. The following years LRRK2 gene mutations and their frequency [17, 18] made the aforementioned gene a major subject of PD research. Despite the thorough study of LRRK2 for over a decade, its physiological role and precise implications in the disease pathogenesis are still the subject of much work.

Leucine-rich repeat kinase 2 (LRRK2) is a large multi-domain protein that belongs to the ROCO protein family, along with death-associated protein kinase 1 (DAPK1), malignant fibrous histiocytoma amplified sequence 1 (MASL1 or MFHAS1) and leucine-rich repeat kinase 1 (LRRK1) in humans. Those proteins are characterized by a sequence of ROC (Ras of complex proteins) - COR (C-terminal of ROC) dual domains. However, the domain topology that surrounds this complex is quite diverse among ROCO proteins [19].

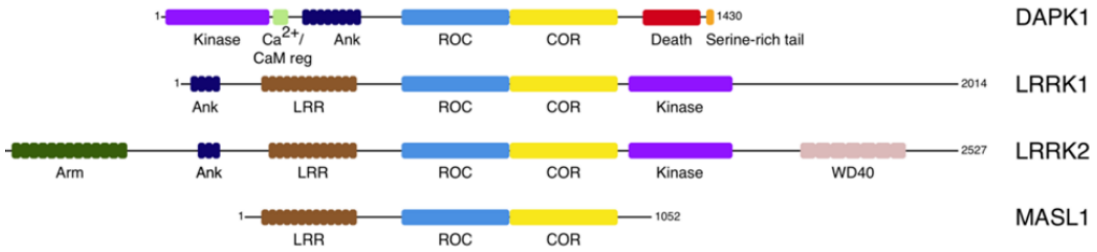

Figure 2: Roco proteins and their domains [19].

LRRK2 mRNA expression is detected in all human tissue tested, while being present in most brain regions, most notably in the substantia nigra and putamen. Its protein expression has been reported mostly in kidney, lungs, liver and heart, but it is also expressed in the brain, predominately in the striatum, cerebral cortex, hippocampus and cerebellum [20, 21]. Furthermore, LRRK2 protein levels have been detected in peripheral blood mononuclear cells (PBMCs), as well as isolated CD14+ monocytes,

CD19+ B-cells and CD4+ and CD8+ T-cells. [22]. LRRK2 does not contain any trans-membrane domains and thus is expected to be a cytosolic protein. However, it is reported to localize in organelle membranes and various membranous structures, where it is most active [23, 24].

LRRK2 is a 286-kDa dual enzyme that exhibits both kinase and GTPase activity. It also contains four distinct domains which are involved in protein-protein interactions, armadillo, ankyrin and leucine-rich repeat (LRR) at the N-terminal and WD40 at the C-terminal of the protein [23]. Among the pathogenic mutations met in familial PD cases, 5 of them are in the ROC domain (N1437H, R1441C/G/H/S), Y1699C in the COR domain, while G2019S and I2020T in the kinase domain [25]. Importantly, these mutations affect the enzymatic activity of LRRK2, suggesting a link of this activity to PD pathogenesis.

1.2.1. Physiological and pathological functions of LRRK2

Since the discovery and the beginning of the characterization of LRRK2, its precise role and function still remains mostly uncertain. By dissecting LRRK2 domain by domain it becomes clear that the amount of possible protein-protein interactions (i.e. its interactome) between LRRK2 and other proteins as well as intermolecular interactions hinders the progress of understanding. Armadillo repeats are present in a variety of proteins and are involved in cadherin based cell adhesion and the Wnt signaling pathway while they mostly are connected with cancer [26]. The Ankyrin repeat is a quite common motif in proteins and is involved in protein-protein interactions that regulate the cell cycle, ion transporters and transcriptional initiators. However because of the sequence variation in the individual repeats, Ankyrin is better characterized by its structure than its function in every individual protein it appears in [27]. The LRR domain is also not well understood in LRRK2. This region is highly phosphorylated at Ser860, 910, 935, 955 and Ser973, while when Ser910 and Ser935 are phosphorylated the interaction between LRRK2 and 14-3-3 can be mediated [28]. However, LRRK2 without Armadillo, Ankyrin or LRR domains, retains its intrinsic kinase activity in *in vitro* systems and in several tissues truncated forms of the protein have been found [29]. The WD40 domain named after WD dipeptide repeats, is very common interactive domain in eukaryotic cells. In a protein, it folds into a β-propeller architecture and is involved in protein-protein interactions or protein-DNA

interactions. Even though it is essential for LRRK2 kinase activity, WD40 doesn't appear to display any enzymatic activity [30].

The ROC-COR domains form extensive inter-domain interactions and thus are treated as a ROC-COR tandem domain. This domain entails both the catalytic properties of LRRK2 GTPase activity and interactions necessary for dimerization of the protein. Prokaryotic homologues have been very beneficial for the understanding of this domain tandem. Furthermore, it is shown that the cycle between monomeric and dimeric form in ROCO proteins is governed by the GTP cycle. In fact, it has been observed that when the *Chlorobium tepidum* ROCO protein is in the GDP state, it is in dimeric form, while in its monomeric form it is in a GTP state. In a nucleotide-free state, it seems to be in an intermediate situation [31]. Since the discovery of LRRK2 and its implications in PD, its kinase domain has been extensively studied. LRRK2 is a Serine/Threonine kinase, as the rest of the ROCO proteins and exhibits both phosphorylation and autophosphorylation activity [32].

In order to analyze the physiological role of LRRK2, several *Lrrk2* KO mouse/rat models have been generated. Those models show no survivability issues and live up to the usual lifespan while being fertile, however histopathological abnormalities in peripheral tissues including lung tissue and kidneys have been observed. In fact, *Lrrk2* KO mice developed accumulation and aggregation of α-synuclein and ubiquitinated proteins in kidneys, as well as decrease in LC3-II, indicating autophagy impairment [33]. It is noteworthy though, that kinase dead models don't seem to affect lung phenotype, suggesting that LRRK2 can also play a role of a protein-protein interaction mediator and not only as a kinase for lung function [34]. In *Lrrk2* KO mice, increased number of hippocampal neuroblasts have been observed, proposing a role of LRRK2 in adult neurogenesis [35]. To further investigate the role of LRRK2, double KO mice for both LRRK2 and LRRK1 have been generated and showed significant age dependent neurodegeneration, in similar areas to PD, while these mice demonstrated autophagy-lysosomal pathway impairment [36]. This suggests some redundancy between LRRK1 and LRRK2 activity, however this finding is contradictory for the expected gain-of-function mechanism associated with autosomal dominant PD. However, proteomic analysis showed little overlap between the two enzymes on the interactomes, depicting distinct functions [37].

Being a kinase, a logical procedure for understanding LRRK2's physiological role is to identify its physiological substrates. However, this task proved to be difficult as most substrates were phosphorylated only in *in vitro* studies. For instance, what is still a common *in vitro* substrate, LRRKtide is a peptide sequence from the ERM protein family of Ezrin, Radixin and Moesin which have not been observed phosphorylated under physiological conditions [29]. Furthermore, tau accumulation is sometimes observed in PD patients with LRRK2 mutations and there is evidence that LRRK2 could phosphorylate tau, but there is not yet a consensus regarding this statement [38].

With a combination of mass spectrometry based phosphoproteomics, genetics and pharmacology, a subset of Rab proteins were identified as both *in vitro* and *in vivo* substrates for LRRK2 [39]. Rabs (Ras-related protein in brain) are small GTPases and regulators of intracellular trafficking. Some of the substrates reported were Rab10, Rab8a, Rab3a and Rab12, while others like Rab7a, were deemed as possible substrates. Rabs however seem to not only be substrates of LRRK2, but also there has been evidence supporting that they have an upstream signaling role, mostly around R7L1/Rab29. In mouse Rab7L1 KO or LRRK2 KO models, similar phenotypes with lysosomal inclusions were observed, while the Rab7L1/LRRK2 genetic module was implicated in various cellular activities including axonal termination, endo-lysososmal trafficking as well as lysosomal maintenance [40].

With mass spectrometry and the use of phospho-specific antibodies, p62/SQSTM1 was identified as another endogenous substrate for LRRK2. p62 is a ubiquitin-binding protein that leads ubiquitinated proteins to degradation through the autophagosome, while also being Lewy Body component. In that study, co-expression of p62 worsened the neuronal death caused by mutant forms of LRRK2 [41].

Mounting evidence suggests that α-synuclein accumulation is mediated in part by endolysosomal dysfunctions. Even though LRRK2 is not abundant in neurons, its kinase activity is increased in iPD patients especially in the nigrostriatal dopamine neurons [42]. Furthermore, LRRK2 localizes in structures that include endosomes, lysosomes and autophagosomes providing evidence for its implication to the disease. In the meantime, Rab proteins regulate trafficking of cargo vesicles to endosomes and then lysosomes [43]. Phosphorylation of Rab5 and Rab10 by LRRK2, inhibits their

function by preventing Rab/GDP-dissociation inhibitor factors binding and ultimately inhibiting membrane trafficking and recycling.

LRRK2 is also tightly liked to pathways regulating inflammation. Polymorphisms in LRRK2 gene have been connected with inflammatory diseases such as inflammatory bowel disease, tuberculosis and higher susceptibility to leprosy [44]. In addition, stimuli such as interferon-γ and lipopolysaccharide (LPS) increase mRNA and protein levels of LRRK2 in immune cells [45, 46]. Microglia activation is a hallmark in brain pathology of neurodegenerative diseases. LPS intracranial injection in mice is shown to induce LRRK2 expression in activated microglia [47]. Furthermore, either LPS or adeno-associated virus mediated overexpression of α-synuclein led to degeneration of dopaminergic neurons in substantia nigra, which is attenuated in *lrrk2* KO rats [48].

1.2.2. Mutations of LRRK2

Patients with PD carrying LRRK2 mutations demonstrate diverse pathology and while loss of dopaminergic neurons in the SNpc was a common histopathological observation among them, Lewy Body pathology, the main hallmark of PD was not always present [49]. Depending on the population 1-30% of PD patients are harboring LRRK2 mutations. Furthermore, both homozygotes and heterozygotes show similar phenotypes, indicating a dominant effect [50].

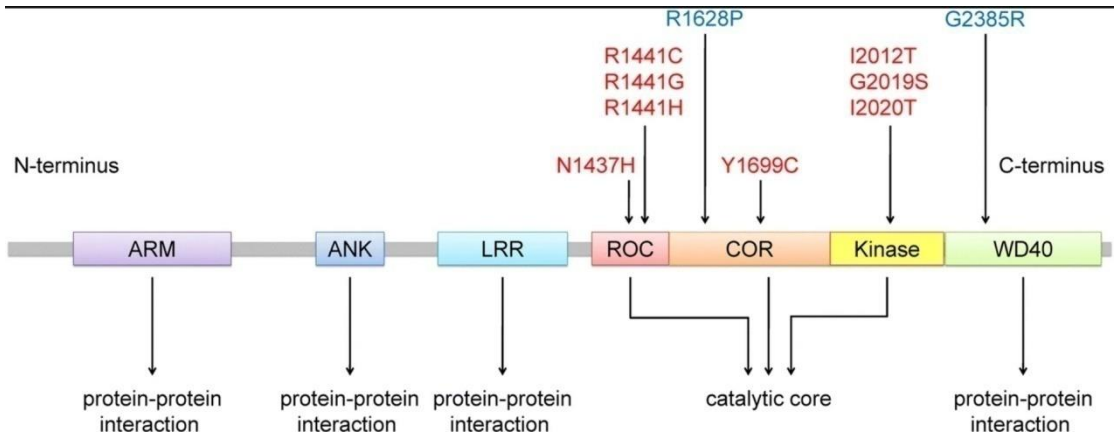

Figure 3: Most common mutations of LRRK2. The vast majority are in the catalytic core [51].

Among all the variations of LRRK2, G2019S, a Serine to Glycine substitution is the most common, while the risk of PD development during a study with 1045 people

with this mutation was 28% at 59 years, 59% at 69 years and 74% at 79 years [52]. This mutation is seen in both familial and idiopathic cases, while its penetrance in PD seems to be age dependent, suggesting the importance of environmental factors to the disease manifestation [53]. Its emergence in apparent idiopathic PD cases is likely due to its incomplete penetrance. Studies over the years have connected G2019S mutation with elevated α-Synuclein and tau protein levels, mitochondrial dysfunction, synaptic vesicle transport disorder, as well as abnormalities in c-Jun, Erk and Akt signaling pathways [54]. *In vitro* kinase activity of recombinant WT and mutant LRRK2 against biotinylated myelin basic protein showed a significant increase in kinase activity of the G2019S mutated protein compared to the WT. In addition, autophosphorylation was also increased in the mutant compared to the WT. Further *in vivo* experiments showed that human G2019S but not the WT LRRK2 delivered by adenoviral vectors in rat brain induced neuronal loss, while inhibition of LRRK2 kinase activity by selective inhibitor PF-360 attenuated this effect [55].

R1441C is another pathogenic mutation of LRRK2. Clinical presentation of R1441C carriers showed similarities with iPD cases as well as with carriers of G2019S mutation. The R1441 amino acid residue is the second most common location of pathogenic LRRK2 substitutions [56]. Along with Y1699C and I2020T, R1441C enhances the association of LRRK2 with microtubules, indicating possible further LRRK2 contribution to neurodegeneration. This effect required intact kinase activity and the presence of WD40 domain. Furthermore, in human post-mortem substantia nigra tissue of people carrying the Y1699C mutation, LRRK2 structures were observed that resembled the microtubule associated filaments [57]. PKA has been reported to phosphorylate LRRK2 at S935 S910, S1443 and S1444 residues, acting as an upstream regulator for LRRK2. 14-3-3 proteins are identified as binding partners of LRRK2, interacting with the pS910 and pS935 residues. R1441C/G/H mutations impair 14-3-3-LRRK2 binding by decreasing phosphorylation of PKA at these residues, while dysregulation of 14-3-3/target protein interaction has been associated with various disorders, including PD [58]. In dopaminergic neurons of *Drosophila*, expression of human WT or R1441C LRRK2 reduced locomotor activity and induced dopaminergic degeneration, creating a PD-like model, while the effect was more robust for the R1441C flies. Proteomic screening indicated downregulation of GTPase activity in the mutated LRRK2 compared to the WT during aging [59]. In further *in*

vivo studies, mice with homozygous R1441C mutation exhibited impairment in motor tasks, olfaction and gait, making it a valid pre-clinical model for prodromal phase of PD [60]. On the other hand, the R1398H LRRK2 variant has shown neuroprotective properties. In contrast to other mutations investigated that showed an opposite effect (R1441G, R1441H and Y1699C) and R1441C that did not seem to have an effect, R1398H increased ROC-COR dimerization, enhancing canonical Wnt pathway. Wnt signaling is considered an essential regulator of neuronal development and maintenance, and indeed the R1398H variant increased axon length [61].

1.2.3. LRKK2 structure and enzymatic activity

LRRK2 is large and complicated protein with many distinct domains and two enzymatic activities. Therefore, it is important to study how conformational dynamics can affect enzymatic activity and vice versa. G-proteins switch between an active GTP- and an inactive GDP-bound state, having highly conserved motifs both for binding α- and β-phosphate of the nucleotide and the interaction with the magnesium ion in the nucleotide binding pocket. Switching between the active and inactive states is regulated by proteins named guanine nucleotide exchange factors (GEFs) by promoting nucleotide release. In the meantime, GTPase activating proteins (GAPs) increase intrinsic GTPase activity and are necessary to switch the protein off. ROCO proteins as well as LRRK2 studied over the years have lower affinity to nucleotides compared to smaller G-proteins and are considered independent on GEFs for activation. Furthermore, LRRK2 is known to be active as a dimer. Studying *Chlorobium tepidum* structure indicated that ROCO proteins that are not able to dimerize, are not able to hydrolyze GTP, proposing that ROCO proteins belong to another class of G-proteins, G-proteins activated by nucleotide dependent dimerization (GADs). In addition, all pathogenic ROC-COR LRRK2 mutations tested either increase the affinity for GTP or decrease the rate of GTP hydrolysis or both which lead to a GTP-bound LRRK2 state. As mentioned before, isolated ROC-COR domain is mostly dimeric in nucleotide free and GDP-bound state, while being monomeric in GTP bound state. It is also important to note that there have not been reported yet homologous interacting proteins engaging with either only the GTP- or the GDP-bound ROC domain, suggesting the possibility that the GTPase activity exists in order to control LRRK2 itself [62].

Protein kinases are molecular switches that catalyze the transfer of γ-phosphate of adenosine tripohosphate (ATP) to a hydroxyl moiety on a protein substrate. They are regulated by two hydrophobic "spines", the regulatory (R) spine, which includes the elements needed for the enzyme activation and the catalytic (C) spine, which is completed by the adenine ring of ATP and is required for the transfer of the phosphate group. In order for the kinase to achieve correct ATP and substrate orientation, as well as transfer the phosphate group, it requires three conserved motifs, the His, Arg, Asp [HRD], the Asp, Phe, Gly plus a hydrophobic amino acid [DFGψ] and the αC helix. In the [DFGψ] motif of LRRK2, Phe is substituted by Tyr and two of the most common mutations (G2019S and I2020T) are located in this motif. Furthermore, it is known that the Y2018P substitution created a hyperactive kinase similar to the familial G2019S variant, deeming the Y2018 residue as a regulator of the enzyme activity, while along with I2020 residue they appear to be critical for keeping LRRK2 in its inactive state [63].

Purified recombinant full-length LRRK2 was analyzed by dynamic light scattering (DLS) and showed that it is primarily dimeric in solution with molecular mass of 581KDa, while Blue Native polyacrylamide gel electrophoresis (BN PAGE) did not detect significant presence of higher oligomeric species. However there are limitations to this technique, such as the inability to distinguish between true oligomers of LRRK2 and LRRK2 bound to other proteins. For this reason, proximity biotinylation method was created of labeling LRRK2 dimers *in situ* [64]. In addition, to study domain-domain interactions, intramolecular chemical cross-linking with mass spectrometry peptide identification, revealed long range according to the primary sequence, interdomain cross-links. N-terminal Ankyrin domain was found in close proximity with C-terminal helix of WD40 and contacts were shown as well between Ankyrin, LRR and kinase domain, indicating a compact protein topology. Furthermore, COR domain displayed lower accessibility, giving further proof that it is involved in the dimerization of LRRK2 [65]. LRRK2 screening based on differential scanning fluorimetry assay that provides stability on multidomain protein complexes in combination with cryo-electron microscopy (cryo-EM), reported further data on domain-domain interactions. In particular, the homodimer protomers are arranged forming an elongating cavity that possibly provides space for protein flexibility and dynamic interactions between the domains, while N-terminal Armadillo domains

curve away from the core. In addition, the dimer is arranged so the one WD40 domain of one protomer interacts with the Ankyrin and LRR domains of the other and the ROC domain of the one protomer is accessible by the kinase domain of the other, not ruling out though intramolecular *cis* phosphorylation [66].

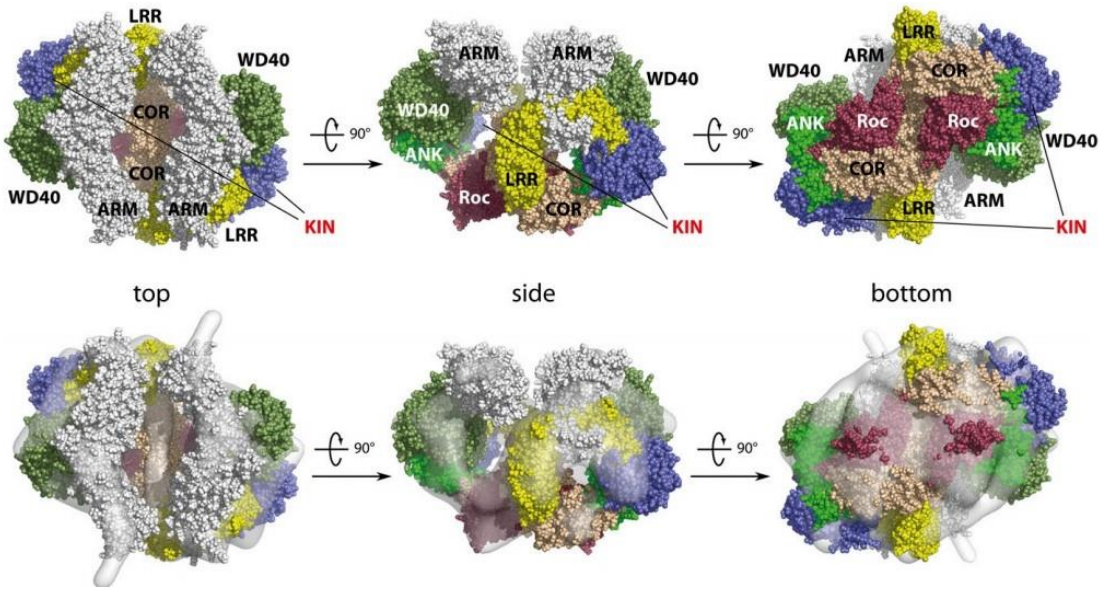

Figure 4: Dimeric 3D LRRK2 model fitted to the EM map [65].

1.2.4. Biomarkers based on LRRK2

Despite the extensive research on PD, there are still no FDA approved biomarkers available for LRRK2 related pathobiology, given its prominent role in multiple forms of PD. Due to the implications of the enzyme and even more the kinase activity of LRRK2 to the disease, the focal point of the current biomarker pursuit has been around the post-translational modifications (PTMs) and activity, focusing on phosphorylation levels of both LRRK2 and its substrates [67]. A first useful indicator that could be variable in different PD cases against control groups, are the total levels of LRRK2 in different cell types/tissues. LRRK2 derived from immune cells is easy to obtain non-invasively, while LRRK2 in various blood cell types is abundant. However, variabilities in levels of LRRK2 in periphery should be treated with caution since LRRK2 is involved in various inflammatory responses and this effect could be PD independent and an important issue to consider in designing such studies is that they are sufficiently powered for the specific statistical tests used. Results on this

topic seem contradictory, since both higher levels of LRRK2 in patients with late-onset PD compared to age matched healthy controls [68] and no different expression of LRRK2 in isolated PBMCS between iPD cases and healthy controls [69] have been reported.

Phosphorylation of LRRK2 may occur either by other kinases upstream or by LRRK2 itself, as autophosphorylaton, but only in the later scenario it would be safe to assume that kinase activity is altered. However, phosphorylations of LRRK2 by other kinases like PKA that was previously mentioned (S910, S935), are much more abundant than autophosphorylations (S1292), so it is difficult to interpret the former PTMs without clear knowledge of their effect on LRRK2 enzyme activity. It has been reported that the over-expression of kinase inactive mutants D1994A and K1906M/R in human embryonic kidney (HEK) 293 cells, showed minimal to no dephoshorylation on S910, S935 and S955 residues after treatment with kinase inhibitors. On the other hand, for the T2035A LRRK2 mutant, massive dephosphorytation of the aforementioned residues was observed upon treatment with inhibitors, at similar levels to the WT LRRK2. These results suggest that LRRK2 dephosphorylation could be independent of its kinase activity [70].Furthermore, pathogenic mutations such as G2019S, R1441C/G/H I2020T and Y1699C that are known to increase kinase activity display reduced phosphorylation of S910 and S935 residues after over-expression in cell lines [71].

Due to its low abundance, not many reports about autophosphotylation sites have been published. In urinary exosomes from G2019S mutation carriers, pS1292 was elevated compared to non-carriers. Furthermore, pS1292 was also detected in cerebrospinal fluid exosomes, suggesting a possible higher LRRK2 activity in brain, than in the periphery [72]. Exosomes are small extracellular vesicles that contain DNA, RNA and proteins that are normally not secreted otherwise, while they can pass the blood-brain barrier, thus they are considered a robust material for biomarker development [73]. Another method to assess LRRK2 kinase activity is by directly measuring it *in vitro* with substrate peptides such as the LRRKtide. In a clinical study using PBMCs from healthy controls, idiopathic and G2019S PD cases, while the total levels of LRRK2 were equal, kinase activity in G2019S carriers was significantly elevated compared to the other groups. In addition, S935 phosphorylation was

elevated in the iPD group compared to the healthy controls and the G2019S mutation carriers [74].

1.3. Nanobodies

Immunoglobulin G (IgG) is an antibody (Ab) isotype derived from B-cells and the most common type in blood. This Y shaped ~150KDa protein is composed of two identical heavy and two identical light chains (HC, LC), with disulfide bridges linking the heterotetramer. Both HC and LC have two domains, the constant (C) and variable (V) domain. Overall, Abs have two fragment antigen domains (Fabs) and a fragment crystallizable (Fc) domain. In the Fab domains, there are two variable sub-domains each from the heavy or light chain (VH, VL) for binding to a specific antigen at the N-terminal of the HC and LC. Furthermore, in the Fab/ VH and VL domains there are six hypervariable regions, complementary-determining regions (CDRs), three from HCs and three from LCs, forming loops and orienting these domains [75]. Despite the plethora of applications antibodies have been undoubtedly used for, there are also intrinsic limitations that hamper their effectiveness as prognostic and therapeutic tools. First of all, due to the numerous disulfide bonds and PTMs such as glycosylations, they become vulnerable to environmental changes. Furthermore, because of their size, shape, affinity and the Fc domain that interacts with various receptors, favorable pharmacokinetics and tissue penetration becomes an issue. Finally, high quality and quantity of Abs require high cost purification steps and production in general [76].

In a study in 1993, considerable amount of novel IgG-like material in the serum of camel (*Camelus Dromedarius*) was identified. This type of IgG consists of heavy chain dimers that are devoid of light chain, while the antigen-binding unit is located at a single variable domain (V_HH) [77]. Thus, by isolating and engineering recombinant V_HHs from *Cameliade* B-cells after immunization, small sized recombinant IgG fragments can be generated. Perfectly describing their size (2.5nm x 4nm, ~13KDa), the term “nanobody” (Nb) was patented by the company Ablynx in 2003 and since 2013 it has been broadly used by the literature [78]. Crystal structures of V_HHs have shown convex surface, CDR1 and CDR2 loops that differ from the canonical structure

of conventional antibodies and a CDR3 loop that is also longer, while kinetics and affinities are approximately at the same range. Interestingly, Nbs seem to have a preference for active sites of enzymes they have been developed against. In a study, out of eight Nbs isolated against hen egg white lysozyme, epitope mapping revealed that six of them were binding into the enzyme's active site cleft [79]. Biophysical and structural characterization of Nbs has shown high thermodynamic and colloidal stabilities with the latter reducing protein aggregation. Furthermore, highly conserved hydrophobic amino acids in the CDR loops found in conventional antibodies are replaced by hydrophilic residues in Nbs, giving them high solubility [80]. In addition, because of their high similarity with human VH sequences, Nbs are considered non-immunogenic [81].

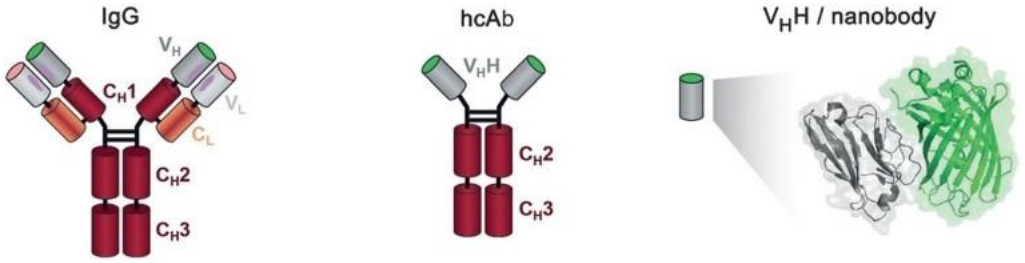

Figure 5: Depiction of IgGs and Ab/Nb size comparison [82].

1.3.1. Generation of Nanobodies

Constructing a Nb phage display library has been established as an efficient and high yield method of Nb production. The Nb production workflow is demonstrated in a study for Nb production against Brucella, a gram-negative coccobacillus, camels were immunized with bacterial lysate to raise immune response and two months later peripheral blood lymphocytes were isolated. mRNA was extracted from those cells, cDNA was prepared via PCR and V_HH fragments were transformed in *E. coli* cells. Then, following phage infection of the *E. coli* bank, viruses were purified and used for panning by incubating in tubes pre-coated with the antigen. After washing off non antigen-specific phages, the eluted particles were used to infect *E. coli* once again and individual colonies were picked and expression of soluble periplasmic Nbs was performed. Finally, the evaluation of the Nbs was tested in ELISA for their affinity for the antigen they were raised against [83].

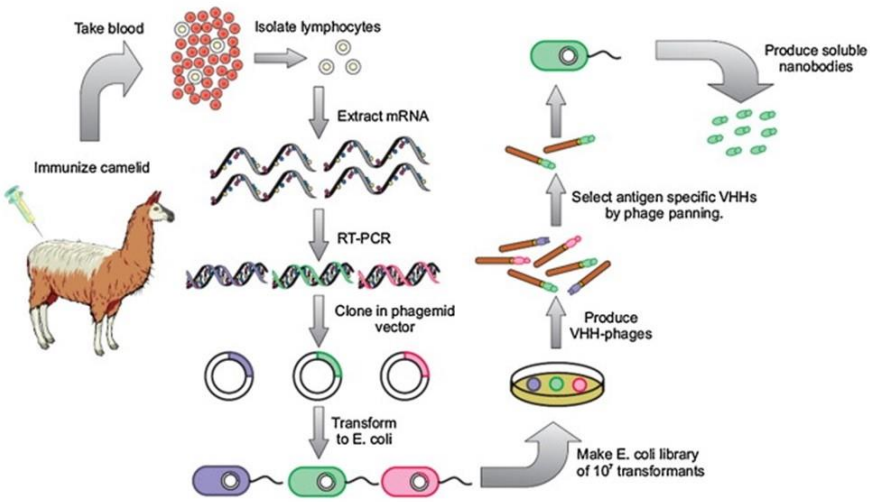

Figure 6: Nb library construction.

1.3.2. Nanobody characteristics and applications

The plethora of advantages of Nbs along with their straightforward generation has led to their consideration as a valuable tool in basic and clinical research. In recent years, Nbs have been used as a surrogate for antibodies in immunocytochemistry. Tagging an organic fluorescent dye directly to a Nb bypasses the need for secondary/tertiary antibody, making the procedure easier and less costly [84]. Since the 1990s, a breakthrough in microscopy has emerged with super-resolution techniques that include stimulated emission depletion (STED), stochastic optical reconstruction microscopy (STORM) or photoactivated localization microscopy (PALM). The need for super-resolution microscopy has come to the surface as diffraction of light, limits observation of structures smaller than ~250nm in the lateral and ~550nm in the axial direction by conventional fluorescent microscopy [85]. Therefore, in super-resolution microscopy, conventional primary/fluorescent secondary antibody reagents lead to high linkage errors, while fluorescent tagged Nbs with diameter of ~2.5x4nm makes them a preferable choice for small subcellular structures. Nbs have also been successfully applied at novel EM protocols. Their small size is beneficial as linker molecules, with improved labeling efficiency and accuracy of gold labeling in immune gold based EM [86].

Fluorescent proteins (FPs) like GFP or RFP are useful biosensors and their fusion to a protein under investigation can reveal its dynamic interactions in living cells. However, this genetic manipulation can affect the function of the protein of interest,

so in order to avoid this possible side effect, co-expression of a FP-tagged Nb (chromobody) could bypass this issue [87]. A different method to study protein-protein interactions is the so called fluorescence three-hybrid assay (F3H). By using an anti-GFP Nb covalently linked to a protein that accumulates at a specific cell location (localization protein/ LP), the GFP tagged protein is recruited at a specific location and by using a different fluorescent label for a second protein of interest, the interaction between these proteins can be observed in real time. In fact, with the use of a single anti-GFP Nb-LP plasmid, any GFP tagged protein can by studied [82]. Alternatively, a co-expression of a "bait" protein equipped with anti-GFP Nb, a GFP-tagged membrane marker and an RFP-coupled "pray" protein can be used to study "bait"-"pray" interactions. When the three constructs interact, exited energy from GFP is transferred to RFP reducing the fluorescence lifetime of GFP, a mechanism called Förster resonance energy transfer (FRET).

Nbs have been successfully applied in MS and as antibody alternatives in affinity-purification mass spectrometry (AP-MS). Their small size minimizes background binding and use of multiple Nb clones for different epitopes in the same antigen can reveal true binders or false positives which can be tackled by a control Nb [88]. In order to determine a protein structure, X-ray crystallography is very beneficial. However, stability of the protein under investigation is crucial, while the production and the quality of the crystals is a challenging aspect of the method. Natural partner proteins can aid the crystal production but not all proteins have such partners that interact strongly with them. With the use of Nbs though, crystallization of a protein can be accelerated with co-crystalization of the complex, providing the protein conformational flexibility [89]. Nuclear magnetic resonance (NMR) spectroscopy is a technique well suited to reveal protein dynamics. In contrast to crystallography, NMR is challenging when studying larger proteins, but due to their small size, Nbs can be implemented as invisible probes, while the target protein is isotopically labeled [90].

Apart from their extensive use in research and diagnostics, Nbs have also earned a place in therapeutics. In early 2019 caplacizumab was the first FDA approved active Nb, an anti-human von Willebrand factor for the treatment of thrombosis [91]. Furthermore, several Nbs have reached preclinical stages, while a number of them are tested in clinical trials, most of them focusing on cancer therapy. Their attributes as small molecules and mAbs make them very appealing for therapeutic target

development. Cancer diagnostics rely on penetration capacity and high specificity, both of which Nbs are able to cover and due to their short half-life in the bloodstream, they provide a desirable tumor to background ratio. Molecular imaging requires Nbs to be labeled with a radioisotope and in a study, the use of anti-HER2 Nb in breast cancer patients showed absence of side effects. In addition, Nbs can be used as drug carriers and as antagonists or allosteric inhibitors, affecting signaling cascades or enzymatic activities of the targeted proteins respectively.

The blood-brain barrier (BBB) is a protective, highly selective, semi-permeable physical barrier that becomes an obstacle in drug delivery as without active transport, it is only permeable for below 400Da lipophilic molecules. Strategies to bypass the BBB use "Trojan horses" that target BBB receptors, resulting though in non-specific passage of other molecules. There have been described Nbs against such receptors that can cross the BBB *in vitro*. However, there have also been reported Nbs with basic isoelectric point against glial fibrillary acetic protein (GFAP) that spontaneously cross the BBB [92].

Nbs against α-synuclein have been generated in order to monitor PD progression or even as therapeutic tools. Specific clones named NbSyn2 and NbSyn87 have been used against monomeric and oligomeric/fibrillar forms of α-synuclein, targeting distinct epitopes of the C-terminal domain. While the C-terminal domain does not seem to be involved in the fibril core, it could potentially stabilize or rearrange oligomeric or pre-fibrillar species during the maturation of the fibrils [93]. These pre-fibrillar transient species that are present at low ratio compared to monomeric α-synuclein, have been characterized either as low-FRET oligomers or as high-FRET oligomers by single molecule FRET (smFRET), while low-FRET oligomers convert to high-FRET during the fibrillar maturation. *In vitro* assays utilizing the NbSyn2 and NbSyn87 were found to slow low to high-FRET conversion and promote a conformational shift to less toxic species [94]. Further *in vivo* experiments took advantage of PEST tagged Nbs, NbSyn87*PEST and V14*PEST that has high affinity for the hydrophobic non-amyloid component domain of α-synuclein which is critical in fibril formation. PEST constructs (proline, aspartate/glutamate, serine, threonine) are proteasomal-targeting motifs that have been shown to stall α-synuclein aggregation when fused to NbSyn87 and VH14, while they mediate proteosomal degradation of α-synuclein. Pathological analysis showed reduced levels of Serine-

129, a highly phosphorylated residue of α-synuclein in LBs and considerable maintenance of striatal dopaminergic neurons, validating Nbs as effective therapeutic tools [95].

In order to produce potent and versatile Nbs, a pivotal step is to label them with suitable probes. Common methods of protein conjugation that are usually applied are not site-specific with the possibility of disturbing the antibody-antigen binding site. On the other hand, the use of Sortase A (SrtA), a transpeptidase derived from *Staphylococcus aureus* can solve this issue. For the transpeptidation a two-step mechanism is required, where SrtA cleaves a peptide bond of an LPXTG (sortag) motif between T and G and then a nucleophile (triglycin-functionalized probe) attacks the acyl-enzyme intermediate leading to a formation of a new peptide bond. For this reaction, only the presence of two short peptides is required, LPXTG at the C-terminal of the Nb and an oligoclycin at the N-terminal of the probe, making the procedure simple and site-specific [96].

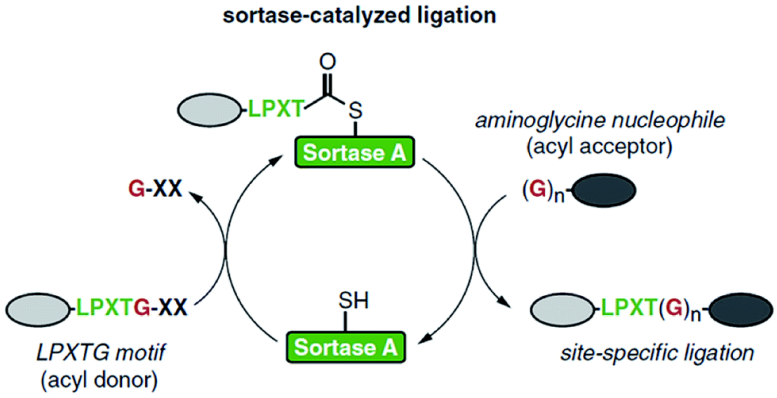

Figure 7: SrtA mediated transpeptidation mechanism [97].

1.3.3. Collaboration with Columbia University

During a previous biomarker study in collaboration with Columbia University, we used a large PD cohort consisting of idiopathic PD, G2019S-LRRK2 with PD, G2019S-LRRK2 without PD and healthy controls, in order to develop and compare LRRK2-targetted biomarkers. ELISA-based assays of LRRK2 levels, phosphorylation and kinase activity showed that in PBMCs of G2019S mutation carriers, kinase activity was higher. However, in some samples LRRK2 levels were not detectable and

in the mean time, it was difficult to identify specific conformations *in vitro*. Therefore, we wanted an alternative approach to quantify LRRK2 in clinical samples and that was by using Nbs against LRRK2.

1.3.4. Collaboration with Vrije Universiteit Brussel (VUB)

Conformation specific Nbs were developed against monomeric or dimeric LRRK2. Under joint collaboration with Wim Versees from VUB, llamas were inoculated with LRRK2/ guanine nucleotide excess combination for the production of anti-LRRK2 Nbs. The rational for this was the idea that GDP promotes a dimeric, while GTP promotes a monomeric LRRK2 state. The clones extracted were evaluated *in vitro* with recombinant full-length or fragments of LRRK2 with surface plasmon resonance and microscale thermophoresis. Finally, selected clones were amplified and sent to BRFAA for further screening and application in biological samples.

1.4. Objective

Our previous work has shown clearly that dimeric LRRK2 is the active kinase species. Further, in a study of clinical samples from a cohort of LRRK2 mutation carriers, purified G2019S mutant LRRK2 possesses significantly elevated kinase activity. Importantly, we have found that this elevated kinase activity is only manifested when in a homo-dimerc state. Thus our hypothesis is that a build-up of homo-dimeric LRRK2 could be associated with the onset of neurodegeneration. As part of a collaboration, a library of Nbs were constructed against different conformations of LRRK2. The main goal of this study is to identify the Nb clones with high affinity to dimeric LRRK2, as this state is more active in PD and exploit possible conformational changes among LRRK2 mutations in order to expand our biomarker tools. Therefore, we screened the clones for affinity with cell-produced LRRK2 and selected specific clones for *in vitro* applications. Furthermore, carriers of the G2019S mutation from the Columbia University cohort, had hyperactive kinase activity, so we tried to correlate this increased activity with differential Nb affinity.

2. Materials and Methods

2.1 Cell lines

HEK293T cells were grown in DMEM (Dulbecoo's modified Eagle medium, Sigma) supplemented with 10% heat-inactivated fetal bovine serum (10% FBS), 5% penicillin/streptomycin. All cells are maintained at 37ºC in 5% CO2.

2.2 Transfection and plasmids

HEK293T cells were transiently transfected in 10cm or 6-well tissue culture plates using 2M calcium phosphate/HBS (pH:7.05). In the experiments plasmids were co-expressed at a ratio of 1:1. In order to obtain LRRK2 dimers for measurement we used proximity biotinylation assay [97]. Briefly, one LRRK2 molecule is fused to a ligase (BirA) and a second with an acceptor peptide (AP). When those two LRRK2 protomers form a dimer, the peptide is biotinylated with ATP presence and the biotinylated dimer can by captured by streptavidin (SA). Thus, for our experiments we used two plasmids of LRRK2 fused with the aforementioned sequences. After ~16hrs the medium was changed to Opti-MEM (Reduced serum medium, no phenol red, ThermoFisher) supplemented with 4% heat-inactivated FBS, biotin depleted, 5% penicillin/streptomycin.

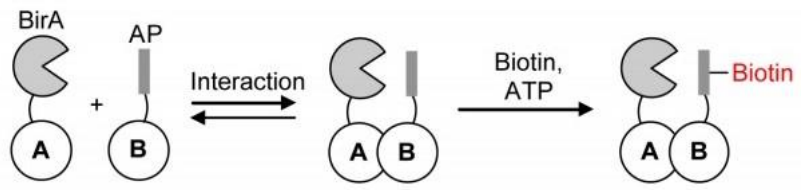

Figure 8: Proximity Biotinylation Assay [64]

2.3 Cell harvest and lysis

48hrs after the biotin depleted media change cells were washed with PBS and were given a pulse with biotin (50μM) for 5min at 37ºC followed by extensive PBS washing. With the biotin pulse BirA/AP LRRK2 dimers were biotinylated. Then, cells were collected with PBS and were isolated by low speed centrifugation. Cells were then lysed with Nonionic P 40 (NP-40) detergent lysis buffer (Tris-HCl 30mM, NaCl

150mM, $MgCl_2$ 5mM, Glycerol 5%, NP-40 0.5%) on ice for 30min and the cell lysate was collected by high speed centrifugation.

2.4 Guanine nucleotide analogs

In order to test the GTP mediated LRRK2 monomerization theory for the first time using full- length LRRK2 we added to selected cell lysates, two non-hydrolysable GTP analogs, GTP-γ-S (Sigma) and GppNHp (abcam) at 1mM.

2.5 Antibodies/ capture agents

Mouse monoclonal anti-Flag antibody (clone M2) was purchased by Sigma. Mouse monoclonal anti-His antibody (J099B12) was from BioLegend. Rabbit monoclonal anti- LRRK2 antibody (UDD3) was from abcam. UDD3 is detecting between amino acids 100-500. Rabbit monoclonal anti-LRRK2 MJFF2 (clone c41-2) was from abcam, detecting a sequence from the 950^{th} residue to C-terminus. Mouse monoclonal anti-LRRK2 (clone N241A/34) was from NIH NeuroMab, detecting a sequence from the 970^{th} residue to C-terminus. Finally, streptavidin was from Sigma. In order to prepare HRP conjugated antibodies, we used HRP conjugation kit by abcam.

2.6 PBMCs from human patients

PBMCs were collected from LRRK2 cohort patients at Dept. of Neurology, Columbia University, NY, USA. PBMCs were isolated using standard protocols. Briefly, sodium citrate or heparin-coated Vacutainer tubes were used to collect whole blood from study participants. Blood was diluted 2X in sterile PBS and transferred to Ficoll-containing Leucosep tubes (Griener) and centrifuged according to the manufacturers' instructions. The banded cells were collected and washed 2X in PBS before counting and aliquoting, in RPMI with FBS and DMSO, at cryovials. Frozen cells were stored at -80ºC. Four groups were included in this project, the control group, iPD group and G2019S LRRK2 patients +/- PD.

2.7 Sandwich ELISA for LRRK2

Various forms of sandwich ELISA took place for this project and they will be seen in detail in later sections so here we will mention only the main principle. First, an Ab was coated overnight in 96-well ELISA plates (Corning Costar) in 100mM NaHCO3

buffer pH: 9.3-9.6 or PBS and after washes with ELISA wash buffer (50mM Trisima-Base, 150mM NaCl, 0.1% Tween-20, pH: 7.4) cell lysate was incubated in 1% bovine serum albumin (BSA) / TBST for 1-2hrs at 37ºC for protein binding. After washing, LRRK2 was detected with horseradish peroxidase (HRP) conjugated antibody (1% BSA/TBST) at RT for 1hr and after a final wash chemiluminescent substrate (ECL, Thermo Scientific) for 5min at RT was added. Every incubation step except of the coating, was performed under constant agitation.

3. Results

3.1. Effect of Guanine nucleotides on LRRK2 dimer levels

It is already established that the prokaryotic Roco protein's and LRRK2 homologue's conformation state, depends on its nucleotide load. When this protein is a nucleotide free or in a GDP-bound state, it is mainly dimeric, while when GTP-bound, it turns to a monomeric state, going through a monomer-dimer conversion via its GDP/GTP cycle. In addition, the rational for specific conformation LRRK2 Nb generation was based on the idea that LRRK2 behaves the same way as the prokaryotic Roco protein.

In order to isolate LRKK2 dimers we took advantage of the proximity biotinylation assay for LRRK2. By using the BirA enzyme fused with WT Flag-LRRK2 and an AP fused with WT c-myc-LRRK2 plasmid, we created a dimer that can be captured by SA when the biotinylation between the two LRRK2 molecules takes place. We coated an ELISA plate overnight with SA and incubated lysate from HEK293T cells transfected with the two plasmids with or without the addition of non-hydrolysable GTP analogs (GTP-γ-S and GppNHp) at 100μM (1hr/37ºC). Then for detection we used Flag (clone M2) antibody conjugated in house with HRP for (1hr/RT) and finally we used chemiluminescent substrate (5min/RT). Here, we demonstrate for the first time that when dimeric full-lengthWT LRRK2 is in an environment with excess of GTP, it is partially forced to a monomeric state, compared to when it is in a nucleotide free state. The effect was stronger with GppNHp. We normalized the results with total LRRK2 levels by using c41-2 clone as capturing Ab and N241/34 as detecting Ab.

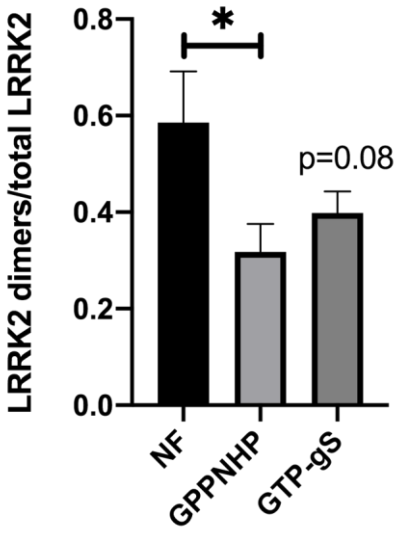

Figure 9: GTP analog effect on LRRK2 dimerization. The first column depicts a nucleotide free (NF) state while the next two the effect of the GTP analogs (GppNHp and GTP-γ-S respectively) on dimeric LRRK2.

3.2. Evaluation of Nb clones based on dimeric LRRK2 affinity

After clone amplification by the VUB, 20 clones were sent to BRFAA for further screening. Here, we also used the proximity biotinylation method and thus, we coated ELISA plates with SA and incubated lysate from HEK293T cells transfected with the two plasmids (1hr/37ºC). For the detection we used the 20 Nb clones, which were 6xHis tagged (1hr/RT) and then we incubated once more with anti-His tag Ab conjugated in house with HRP (1hr/RT) and measured the individual Nb affinity with chemiluminescence. We set the threshold of positive signal for dimeric WT LRRK2 at two times the background ratio of the assay. For this experiment we had 3 biological replicates with highly similar results. We observed consistent positive signal in 9 out of the 20 clones and we refer to them SA positive (SA+) clones.

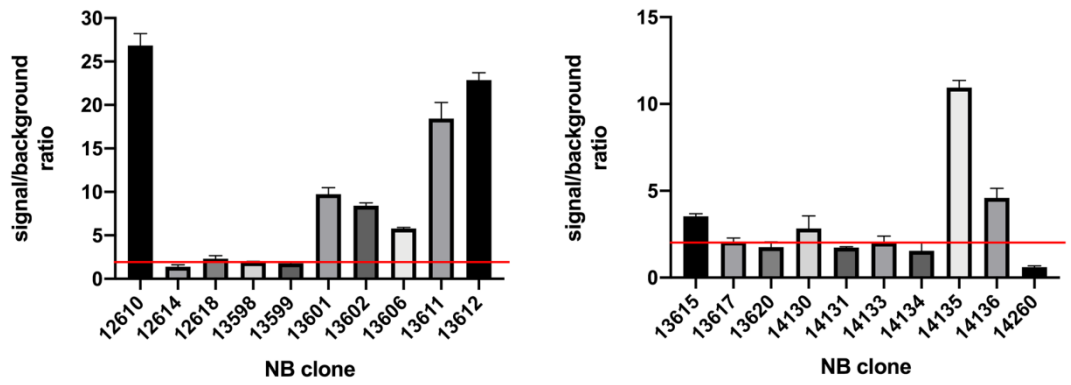

Figure 10: Affinity of Nb clones to LRRK2 dimers.

3.3. LRRK2 dose response and Nb linearity

In order to determine the linearity of the affinity of the SA+ clones, we repeated the same assay in various total protein amounts of HEK293T/WT LRRK2 cells (0.625µg, 1.25µg, 2.5µg, 5µg and 10µg). Indeed, all clones tested demonstrated good linearity for the protein concentrations we used in the study. Usually in ELISA in order to characterize and validate the linearity of an Ab, recombinant protein is used, however here we do not have this option, since different Nb clones for different LRRK2 species have different affinities. So, we did a dose response experiment with increasing amounts of protein extract.

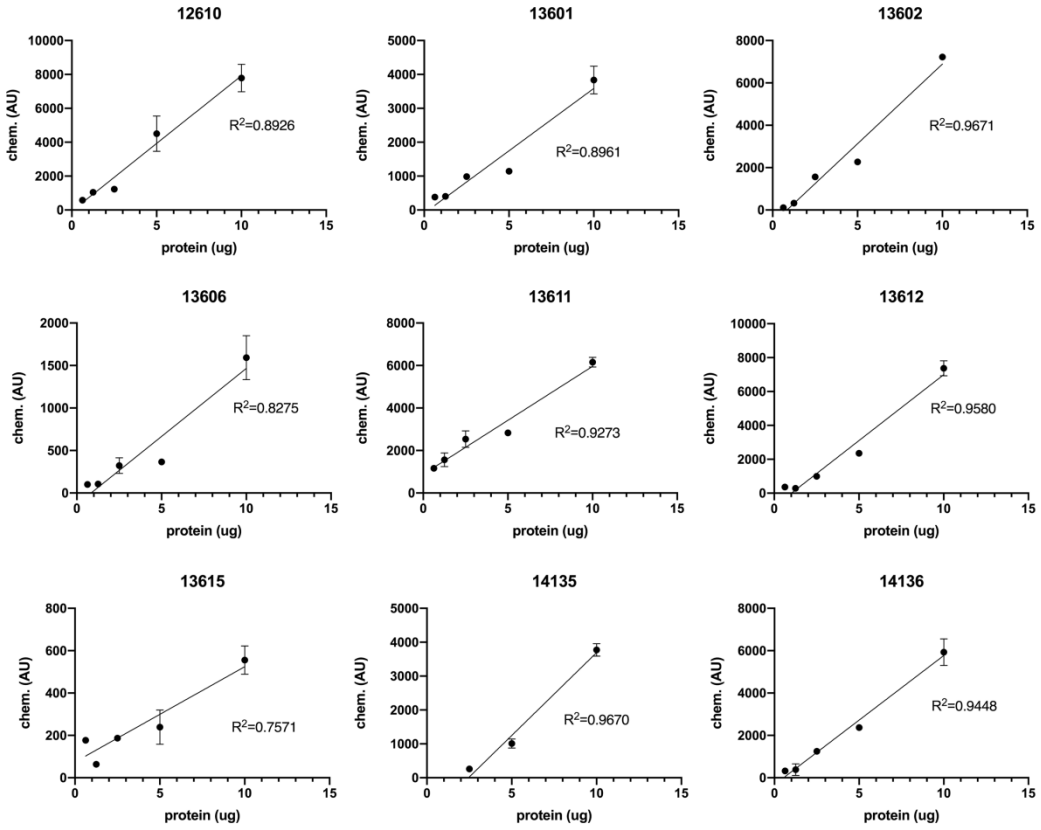

Figure 11: Dose response of SA+ clones.

3.4. Nb affinity for mutant LRRK2 dimers

A current question about LRKK2 mediated PD is how LRRK2 mutant dimers contribute to the disease. Nb's ability to have conformational affinity for an epitope could possibly allow us to detect changes between different LRRK2 mutations. For this experiment, we performed the proximity biotinylation assay for G2019S LRRK2, as well as R1441C LRRK2 with appropriate BirA or AP fused constructs. In that way, we could compare how alterations within the mutated LRRK2 dimers could affect the affinity of the clones. It has been shown that Nbs usually react with the catalytic core of an enzyme, when they are raised against one and the vast majority of LRRK2 mutations are indeed located in the ROC-COR or kinase domain, with G2019S been in the kinase and R1441C in the ROC domain. In addition, the way the proximity biotinylation method is set, allowed us to also use both homodimeric and heterodimeric constructs, G2019S/G2019S and WT/G2019S as well as R1441C/R1441C and WT/R1441C, as both spices could play a role in the disease

progression. Furthermore, G2019S and R1441C-LRRK2 mutations are directly linked with PD manifestation and progression.

For the ELISA in this part, we followed the same steps like previously with the difference that apart from HEK293T/ WT LRRK2 lysate, we also included G2019S/G2019S, WT/G2019S, R1441C/R1441C and WT/R1441C. We normalized the protein levels with total LRRK2 levels by using c41-2 clone as capturing Ab and N241/34 as detecting Ab.

Here, we observed statistical significance in 2 of the clones tested (13611 and 13606) when comparing WT LRKK2 with homodimeric G2019S LRRK2. In fact, 13611 had an increased affinity for G2019S/G2019S LRRK2 form compared to the WT, while 13606 had the opposite effect. On the other hand both 13611 and 13612 had significantly higher affinity for both homo and heterodimeric R1441C LRRK2 compared to the WT.

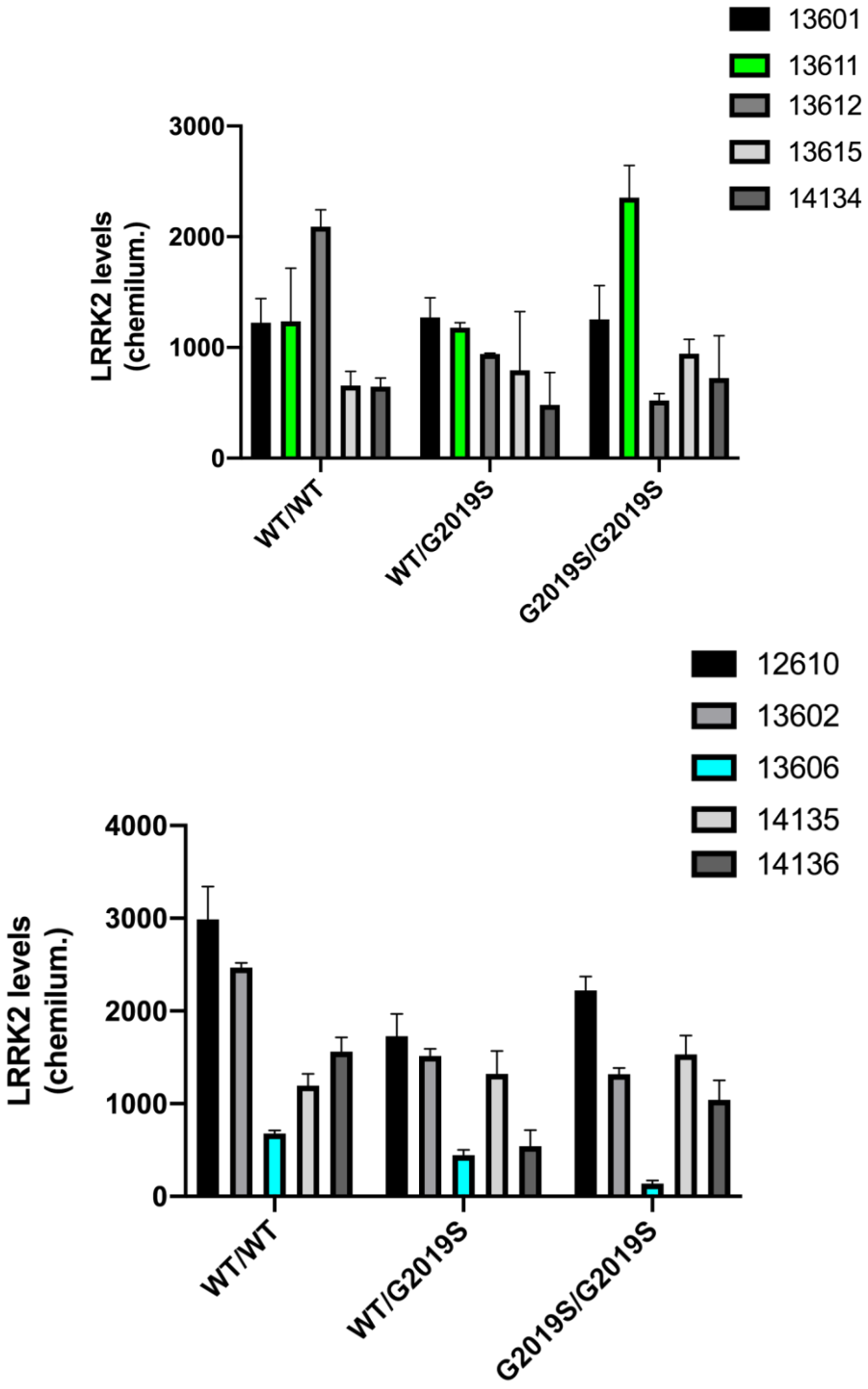

Figure 12: Affinity of selected Nb clones against dimeric WT, WT/G2019S and G2019S/G2019S LRRK2.

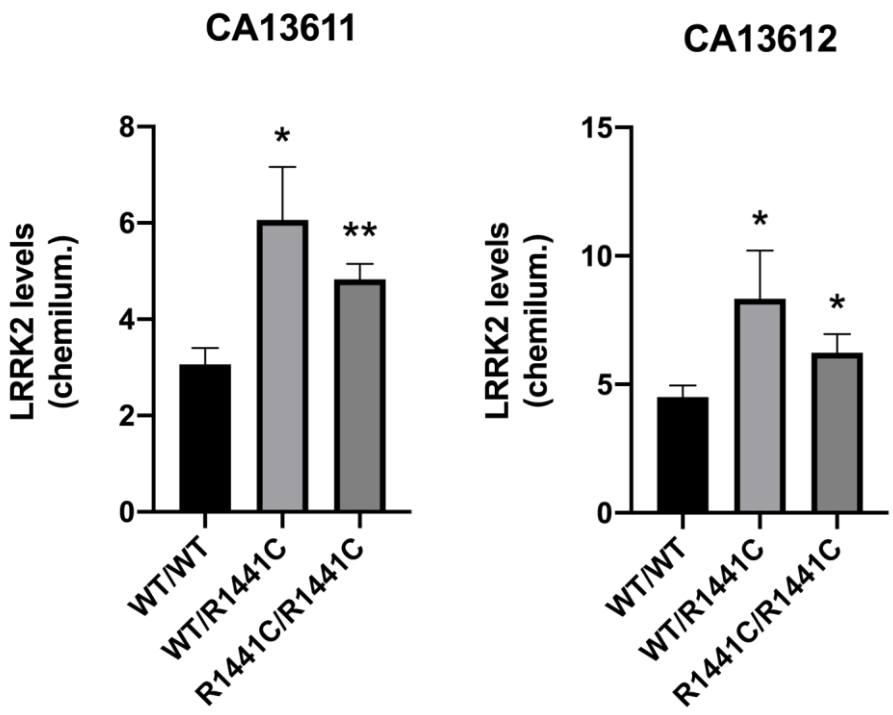

Figure 13: Affinity of selected Nb clones against dimeric WT, WT/R1441C and R1441C/R1441C LRRK2.

3.5. Application of Nbs on human PBMCs

In order to evaluate that the Nbs we have selected are applicable as potential biomarkers in human sample, we tested them for LRRK2 affinity in human PBMCs. Based on differential affinity of some Nbs for mutant LRRK2 we examined clinical samples for Columbia Univ. cohort including carriers of G2019S LRKK2 mutation. From the samples used in this experiment, 5 of them where from healthy controls, 5 from iPD patients, 5 from G2019S LRRK2 +PD and 6 from G2019S LRRK2 -PD patients. Thus, considering the small sample group these experimental results should be considered preliminary and a bigger sample size is required in order to deduce safer assumptions.

This sandwich ELISA set us is quite different from the ones preceded this experiment. The coating was performed by using the Nb clones selected for this assay in $NaHCO_3$ at ph: 9.3-9.6 and left overnight. After washing with ELISA wash buffer, 21 samples were incubated for 2hr in 1% BSA/TBST at 37^{o}C, in a randomized manner in order to

avoid biases in the statistical analysis. As detection Ab we used UDD3 conjugated with HRP in house and the assay was measured with chemiluminescence. We normalized the measurements with total LRRK2 levels by using c41-2 clone as capturing Ab and UDD3 as detecting Ab.

First, by using c41-2/UDD3 Ab combination we observed no significant total LRRK2 expression level differences among the groups.

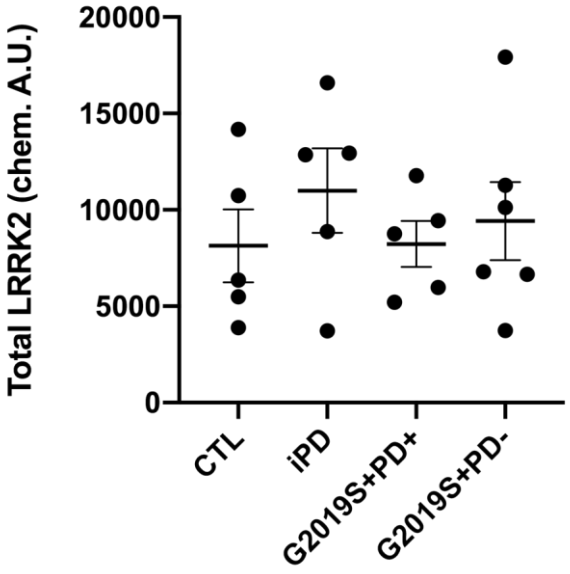

Figure 14: Total LRKK2 expression levels.

In order to interpret the results from the clones selected, we could either normalize to the total levels or just evaluate the raw signal, especially now that total levels seem rather equal. The normalization way seems as more appropriate, but considering the fact that different Nb clones have higher affinity for different LRRK2 species, this is also not something optimal, so here we present both ways.

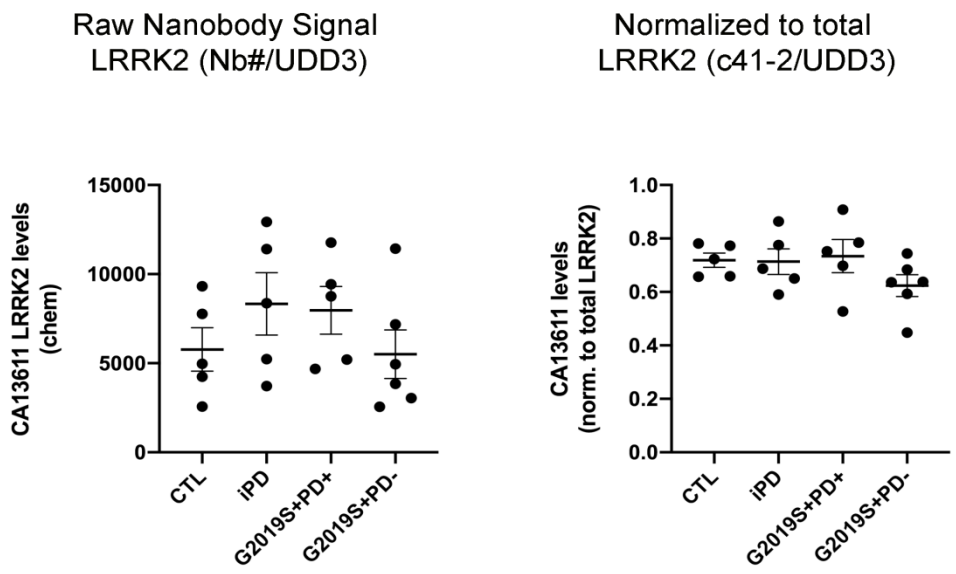

Figure 15: Nb affinity to human PBMC samples (clone 13611).

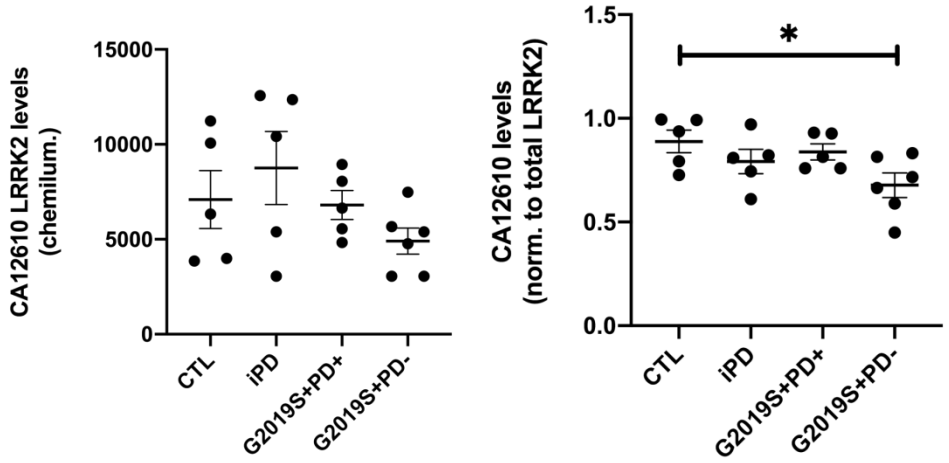

Figure 16: Nb affinity to human PBMC samples (clone 12610).

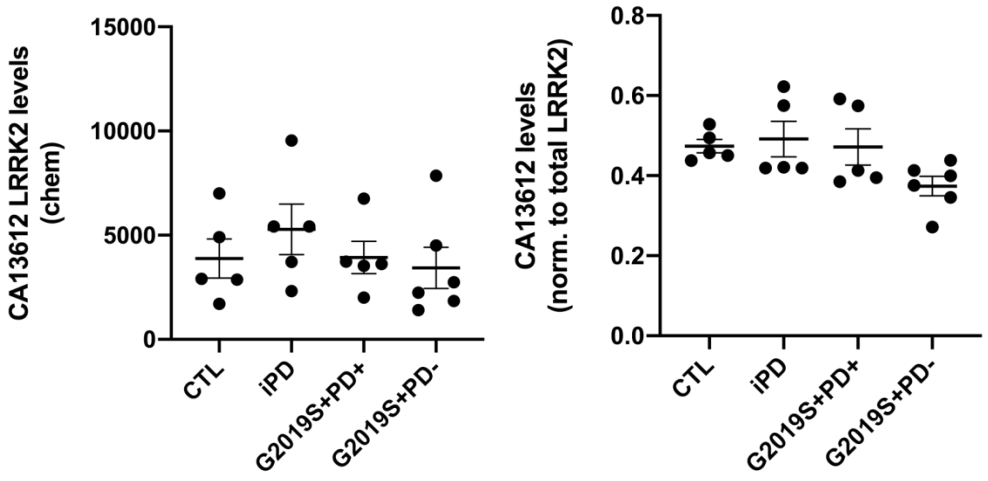

Figure 17: Nb affinity to human PBMC samples (clone 13612)

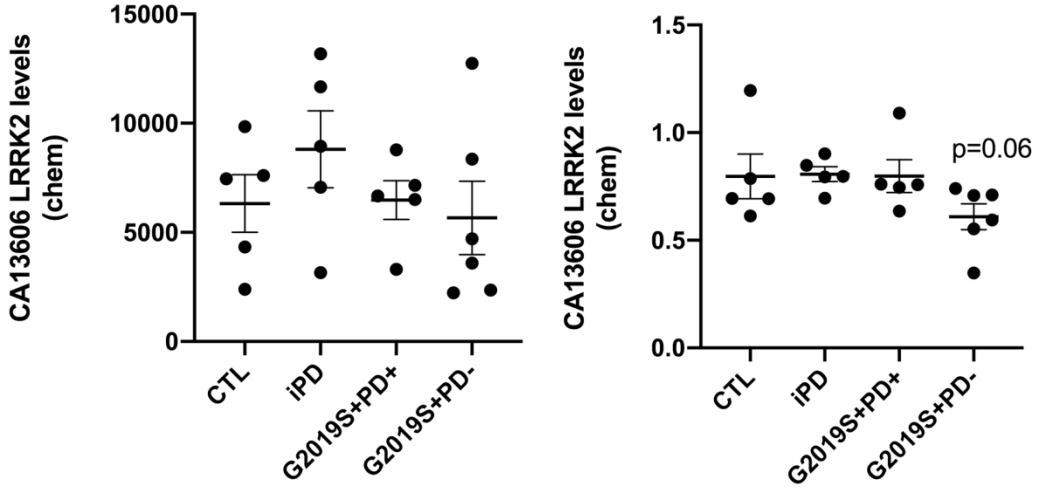

Figure 18: Nb affinity to human PBMC samples (clone 13606).

Here, we observed a trend towards higher affinity for the two PD+ groups compared to the control and PD- for 13611 in the raw data (figure 15). This comes to an agreement with the cell line *in vitro* results with 13611 having a higher affinity for G2019S LRRK2 homodimers. It is still uncertain if G2019S LRRK2 mutation carriers or iPD patients have higher dimeric LRRK2 levels but this result could possible hint a connection between the disease and LRRK2 dimerization status. The 12610 clone

showed a statistical significant decrease in affinity for the G2019S PD- mutation carriers compared to the healthy controls when normalized to total LRRK2 levels, which is still hard to interpret (figure 16). The 12612 clone did not show any variability among the groups either with the raw or normalized signal, showing possibly a neutral marker that does not show variations during the disease progression (figure 17). Finally the 13606 clone contrary to what we were expecting from the HEK293T/LRRK2 protein extract that showed reduced lower affinity for G2019S LRRK2 homodimers, showed a trend for higher affinity for the iPD group in the raw and an almost significant decrease in the normalized results for just G2019S PD- group compared to the rest (figure 18).

Concluding, combining the different assays 13611 clone might be specific for PD or mutation status and could be a candidate disease tracker. However, this is still a trend and a larger sample size could improve or clarify both 13611's as well as the rest of the clones' affinity for different LRRK2 species.

4. Discussion

Deeper understanding of LRRK2 mediated PD which is seen in both familial and idiopathic PD patients is still in early stages, despite the plentiful research that has been accomplished to date. The development of reliable biomarkers that can predict the disease's progression or even detect PD during the pre-motor stages is critical for both earlier diagnosis and molecular mechanism elucidation. In a previous study, collaborating with Columbia University, we observed that in PD patients harboring the pathogenic G2019S mutation kinase activity of the purified enzyme from PBMCs, was elevated even in patients that did not manifest the disease. Here, we took advantage of the conformational specificity of Nbs and proceeded to apply them as possible biomarker tools and Ab surrogates. By screening 20 Nb clones against dimeric WT LRRK2 from HEK293T cell extract, we found 9 clones with relatively higher affinity for these species. Then, we used those results to screen for differences in affinity between dimeric WT LRRK2 and the pathogenic G2019S and R1441C LRRK2 mutants. Finally, we selected specific clones for applications in human PBMCs.

At first we wanted to confirm that LRRK2 undergoes the same GTP cycle as its prokaryotic analog ROCO. The way anti-LRRK2 Nbs against either monomeric or dimeric LRRK2 were generated by our collaborator Wim Versees, was based on this idea, but still there was no definitive answer. However, here we demonstrate for the first time that full-length WT LRRK2 is preferably in a monomeric state when excess of GTP is present.

The fact that dimeric LRRK2 is mostly associated with kinase activity, made us focus more on the clones with higher affinity for this conformation. For this, we heavily relied on the proximity biotinylation assay in order to label LRRK2 dimers *in situ* and used the strong biotin-streptavidin bond to capture and then measure and evaluate the Nb clones that were provided to us. However, a limitation of this assay is that we were not able to measure isolated monomers and use this information as well for biomarker-related purposes. This is why we refrain to claim that the clones with high affinity for biotinylated dimeric LRRK2 are dimer-specific and we call them SA+ clones instead. The clones we selected may also have some minor affinity for monomeric LRRK2, however in this study we focused primarily on their relative

dimer affinity. By overcoming this limitation in future evolutions of this assay, we could characterize with more certainty the specific species each clone has affinity for, something that we aim to address in future studies.

To confirm the linearity in increasing LRRK2 protein levels we used transfected HEK293T derived LRRK2 extract. The usual method of Ab linearity evaluation in any new ELISA involves making a linear curve with recombinant protein. However, Nbs could not allow that, as different clones have preference for different conformational states.

LRRK2 mutants are correlated with altered enzyme activity, mostly increased kinase activity by the most common G2019S mutation, which has a hyperactive kinase domain both *in vitro* and *in vivo*. Therefore, using Nbs to specifically recognize different LRRK2 species related to the disease could be very beneficial for future research. Here, we discovered 2 clones with significantly different affinities for dimeric G2019S and R1441C LRRK2 in HEK293T cells compared to the WT. In fact clone 13611 showed higher affinity for homodimeric G2019S dimers compare to both heterodimeric (WT/G2019S) and WT forms. In addition, it had higher affinity for both the heterodimeric (WT/R1441C) and homodimeric R1441C mutation. In contrast, clone 13606 had reduced affinity for homodimeric G2019S compared to both heterodimeric (WT/G2019S) and WT forms, while clone 13612 had higher affinity for both the heterodimeric (WT/R1441C) and homodimeric R1441C mutation. This is a very interesting finding, and could possibly indicate a large variety of LRRK2 conformations among the different pathogenic mutations. The implications of this are unclear since there appears to be no clinical difference between carriers of the different LRRK2 mutations. Different conformations of LRRK2 dimers could lead to phosphorylation of a different pool of substrates, an altered regulation of LRRK2 by phosphatases, or an altered localization of LRRK2 itself.

Finally, we used the 2 clones with differential affinity for G2019S LRRK2, (13611 and 13606) along with 2 others (12610 and 13612) to measure different LRRK2 species in clinical samples in human PBMCs. The cohort was the same as our previous study that observed higher kinase activity in G2019S carriers, so we were testing for possible correlations between dimeric LRRK2 conformations and a hyperactive kinase. Nb clone 13611 showed a trend that correlated its conformation

specificity with PD manifestation, with both iPD and G2019S PD+ carriers had higher raw signal than healthy controls and G2019S PD- carriers. Interestingly, clone 13606 showed the different results from what we expected from the cell line *in vitro* results as the G2019S PD- group showed almost significant decrease in affinity, while the G2019S PD+ group did not show any difference in the affinity. Clone 12610 showed significantly lower affinity for the G2019S PD- group compared to healthy controls, while clone 12612 did not show affinity alterations in this small sample.

However, the sample size was quite limited so a larger scale follow-up study with more samples added could increase the reliability of the method. In addition, we intend to add more groups, especially the mutant α-synuclein (A53T) carrier group, which is also more common in Greece. Our ultimate goal for a Nb-driven LRRK2 biomarker study, would be to isolate clones that are more sensitive for certain protein conformations, or clones that change their affinity during the disease progression. It is possible that during disease onset and as the disease progresses, different LRRK2 species are prevalent, and thus identifiable by distinct Nb clones.

For that purpose, in future studies we aim to employ other assays as well to evaluate Nb affinity for specific LRRK2 species, other than ELISA. One possibility is by using Fourier-transformed infrared spectroscopy (FTIR) or cryo-EM with Nbs binding to LRRK2 to reveal more detailed structural information and obtain subtle differences that could be crucial for the LRRK2 field; especially in the area of therapeutic development. For example, one potential outcome could be a unique class of inhibitors that bind distinct LRRK2 dimeric species associated with specific disease states. In conclusion, while we still do not completely understand how LRRK2 different species associate with PD manifestation and progression, based on their conformational specificity, we see Nbs as a powerful tool to address these questions.

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