PROF. OMAR EL-AGNAF (Orcid ID : 0000-0002-6850-8084)

Article type : Review

Antibodies against alpha-synuclein: tools and therapies

Nishant N. Vaikath[1], Issam Hmila[1], Vijay Gupta[1], Daniel Erskine[2], Martin Ingelsson[3], Omar M. A. El-Agnaf[1]

[1]Neurological Disorder Research Center, Qatar Biomedical Research Institute (QBRI), Hamad Bin Khalifa University (HBKU), Qatar Foundation, PO Box 5825, Doha, Qatar
[2]Institute of Neuroscience, Ageing Research Laboratories Newcastle University, Campus for Ageing and Vitality, Newcastle upon Tyne, NE4 5PL, United Kingdom
[3]Uppsala University, Department of Public Health/Geriatrics, Dag Hammarskjölds vag 20, Uppsala, SE 75185

Corresponding author:
Omar El-Agnaf
Qatar Biomedical Research Institute (QBRI),
Hamad Bin Khalifa University (HBKU), Qatar Foundation,
Doha, Qatar
Email: oelagnaf@hbku.edu.qa
Phone: +97455935568

Abstract

Synucleinopathies including Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) are characterized by the abnormal accumulation and propagation of α-synuclein (α-syn) pathology in the central and peripheral nervous system as Lewy bodies or glial cytoplasmic inclusions. Several antibodies against α-syn have been developed since it was first detected as the major component of Lewy bodies and glial cytoplasmic inclusions. Over the years, researchers have generated specific antibodies that alleviate the accumulation of intracellular aggregated α-syn and associated pathology in cellular and preclinical models of synucleinopathies. So far, antibodies have been the first choice as tools for research and diagnosis and currently, a wide variety of antibody fragments

have been developed as an alternative to full-length antibodies for increasing its therapeutic usefulness. Recently, conformation specific antibody-based approaches have been found to be promising as therapeutic strategies, both to block α-syn aggregation and ameliorate the resultant cytotoxicity, and as diagnostic tools. In this review we summarize different α-syn specific antibodies and provide their usefulness in tackling synucleinopathies.

Abbreviations

α-Syn – Alpha-synuclein
AD – Alzheimer's disease
CSF – cerebrospinal fluid
DLB – dementia with Lewy bodies
ELISA – enzyme-linked immunosorbent assay
Fab – Fragment antigen-binding
Fc – Fragment crystallizable region
GCI – glial cytoplasmic inclusion
IgG – immunoglobulin G
kDa - kilodalton
MSA – multiple systems atrophy
NAC – non-amyloid component of plaques
o-α-syn – oligomeric alpha-synuclein
pS129-α-syn – alpha-synuclein phosphorylated at serine 129
PD – Parkinson's disease
PDD – Parkinson's disease dementia
scFv – single chain fragment variable
t-α-syn – total alpha-synuclein
VH – heavy variable domain
VL – light variable domain

Introduction

The synucleinopathies are a group of neurodegenerative disorders characterized by the abnormal accumulation and cellular deposition of the protein alpha-synuclein (α-syn). This spectrum of neurodegenerative disorders includes Parkinson's disease (PD), Parkinson's disease dementia (PDD), dementia with Lewy bodies (DLB) and multiple systems atrophy (MSA) (Spillantini & Goedert 2000). Collectively, the synucleinopathies are second only to Alzheimer's disease in prevalence and there is no disease-modifying therapy for these disorders. However, better understanding and improved therapeutics for the synucleinopathies is a healthcare priority as they are associated with increasing age, meaning that their incidence is expected to rise given sustained global population aging.

The synucleinopathies can be further sub-divided into Lewy body diseases, including PD, PDD and DLB, which are characterized by the accumulation of α-syn into spherical lesions within neuronal soma called Lewy bodies and in neuronal processes as Lewy neurites (Spillantini *et al.* 1997). Lewy pathology forms in a stereotyped sequence within the brain, with such pathological progression thought to underlie clinical progression. In contrast, the typical lesions observed in MSA are α-syn accumulations within oligodendroglia, termed Papp-Lantos bodies or glial cytoplasmic inclusions (GCI), and lack the distinct spherical structure of Lewy bodies (Spillantini *et al.* 1998a).

Parkinson's disease is the most prevalent synucleinopathy and is clinically characterized by cardinal motor features of bradykinesia, rest tremor and postural instability that are partially ameliorated by dopaminergic therapy (Gelb *et al.* 1999). Cognitive decline often follows motor dysfunction in PD, and is clinically manifest as dementia in 30% of cases (Hanagasi *et al.* 2017). PDD is clinically characterized by executive and attentional deficits, often along with visuo-perceptual abnormalities and hallucinations (Aarsland 2016). In contrast to PD, where motor dysfunction precedes cognitive decline, DLB patients initially present with cognitive symptoms similar to PDD, including executive and attentional deficits and prominent complex visual hallucinations (McKeith *et al.* 2017). Motor dysfunction is not universal amongst DLB patients but occurs in parallel with or at a later stage than the cognitive symptoms (McKeith *et al.* 2017). There is a continued debate as to whether PDD and DLB are the result of same pathological process or whether these disease entities rather should be seen as different manifestations within the same spectrum (Lippa *et al.* 2007). It has also been suggested that the distinct sequence of clinical features in PDD and DLB may result from pathology originating at different predilection sites, with PD beginning in the motor regions of the brainstem and midbrain, perhaps subsequent to peripheral α-synucleinopathy, and DLB originating in the olfactory bulb interconnected to cognitive regions of the limbic system and neocortex (Rey *et al.* 2018).

MSA is the third most common synucleinopathy diagnosed in patients at an age of around 60 years. MSA is relatively rare compared to PD and DLB though is often mis-diagnosed as PD, especially during the early stages of the disease. However, MSA typically follows a more aggressive course than PD with marked autonomic symptoms and usually a poor response to dopaminergic medications (Hughes *et al.* 1992).

α-Synuclein

α-Syn was originally identified in purified preparations of cholinergic vesicles from the electric organ of the electric ray from the genus *Torpedo* (Maroteaux *et al.* 1988). Initial studies on α-syn in neurodegenerative disorders were focused on AD, where high levels of a fragment of α-syn corresponding to amino acids 61-95 now termed the non-amyloid component of plaques (NAC), was reported in senile plaques (Ueda *et al.* 1993). α-Syn was first associated with the disorders now termed synucleinopathies when a kindred with familial PD originating from the Italian village of Contursi Terme was reported to have a point mutation in the α-syn gene *SNCA* (Polymeropoulos *et al.* 1997). Following this original paper, Spillantini and colleagues then reported the presence of α-syn in Lewy bodies in PD and DLB (Spillantini *et al.* 1997) and later in Papp-Lantos bodies/GCI in MSA (Spillantini *et al.* 1998a). Since then, further genetic studies have confirmed point mutations in *SNCA* (A53T, A30P, E46K, H50Q or G51D) (Kruger *et al.* 1998; Polymeropoulos *et al.* 1997; Zarranz *et al.* 2004; Appel-Cresswell *et al.* 2013; Lesage *et al.* 2013) and duplications/triplications in *SNCA* (Singleton *et al.* 2003; Chartier-Harlin *et al.* 2004; Ross *et al.* 2008) in familial PD. Polymorphisms in the *SNCA* promoter confers increased risk of idiopathic PD (Pals *et al.* 2004), polymorphisms in *SNCA* have been linked with PD risk (Kay *et al.* 2008; Ritz *et al.* 2012) and drugs that decrease α-syn transcription are associated with a reduced risk of PD (Mittal *et al.* 2017). In summary, there is compelling evidence that α-syn has a central role in synucleinopathies.

α-Syn exists in a dynamic equilibrium between a soluble and membrane-bound state and the interaction with lipid surfaces is believed to mediate its cellular functions (Burre 2015). In the cytosol, α-syn is an intrinsically unstructured monomer with a natively unfolded state (Weinreb *et al.* 1996; Kim 1997; Fauvet *et al.* 2012a; Burre *et al.* 2013) . α-Syn was later reported to exist as a metastable tetramer (Bartels *et al.* 2011; Wang *et al.* 2011), though other studies reported contrary evidence (Fauvet *et al.* 2012b; Burre *et al.* 2013; Binolfi *et al.* 2012; Smaldone *et al.* 2015) and the formation of multimers is now thought to result from α-syn interacting with lipid membranes (Burre *et al.* 2014) (Fig. 1).

α-Syn aggregation

The aggregation of α-syn from a disordered monomer to highly organized fibrils is strongly implicated in the pathogenesis of synucleinopathies. *In vitro* studies suggest that aggregation of α-syn is a nucleation-dependent process characterized by an initial lag phase, followed by a growth phase known as “elongation” (El-Agnaf *et al.* 1998; Wood *et al.* 1999;

Cremades *et al.* 2012). During the lag phase, there is an accumulation of oligomers (soluble species without fibrillar conformation) which, over time, develop into fibrils (El-Agnaf *et al.* 2003b; Paleologou *et al.* 2005; El-Agnaf *et al.* 2006; Paleologou *et al.* 2009; Tokuda *et al.* 2010). There is converging evidence to indicate that intermediate oligomers or protofibrils are particularly cytotoxic (El-Agnaf *et al.* 2003a; Winner *et al.* 2011; Helwig *et al.* 2016), and that late stage Lewy bodies, composed primarily of α-syn fibrils, may be less toxic (Bengoa-Vergniory *et al.* 2017; Caughey & Lansbury 2003). Such a proposition is consistent with neuropathological studies reporting widespread Lewy body pathology without clinically significant motor or cognitive symptoms (Frigerio *et al.* 2011; Parkkinen *et al.* 2005) and the lack of relationship between severity of Lewy body deposition and important clinical variables such as disease duration or severity (Colosimo *et al.* 2003; Parkkinen *et al.* 2008).

Despite the evidence that oligomeric α-syn is particularly neurotoxic, *in vitro* studies have also reported that pre-formed α-syn fibrils can also seed aggregation of monomeric α-syn and are cytotoxic to cultured neurons (Luk et al, 2012; Pieri et al, 2012; Prusiner et al, 2015; Peelaerts et al, 2015). It may be the case that oligomers exert their toxicity by further developing into fibrillar forms, or that there are a diversity of α-syn aggregates of differing cytotoxicity. Therefore, it remains unclear as to which strain of α-syn is most toxic, with the lack of characterization of distinct strains of α-syn impeding efforts to address this question. Nevertheless, *post-mortem* studies have reported that the abundence of Lewy bodies composed of tightly aggregated α-syn fibrils are predictive of important clinical variables such as disease duration or severity (Colosimo et al., 2003; Parkkinen et al.,2008). Whilst there is compelling evidence for a central role for α-syn in synucleinopathies, Lewy bodies may not be directly responsible for eliciting clinical features but may rather be a useful neuropathological marker of a process that produces cytotoxic aggregates (Ansorge *et al.* 1997; Milber *et al.* 2012).

The growing realization that Lewy body severity relates poorly to important clinical and pathological variables has stimulated interest in the search for α-syn proteoforms that may be more relevant to the disease process, with considerable interest focused on oligomeric forms that precede the formation of fibrils. Oligomeric α-syn has been observed in degenerating neurons of PD patients, tying it more directly to neuronal degeneration than Lewy bodies (Sharon *et al.* 2003; Tofaris *et al.* 2003). A direct link between α-syn oligomer exposure and cell death has been demonstrated with *in vitro* studies reporting a direct relationship with cell death (Chen *et al.* 2007; Danzer *et al.* 2007; Nasstrom *et al.* 2011). Studies in model organisms have also demonstrated that oligomeric α-syn is capable of inducing cell death,

with dopaminergic neurons especially vulnerable (Karpinar *et al.* 2009; Winner *et al.* 2011; Outeiro *et al.* 2008; Dimant *et al.* 2013). The exact mechanism of α-syn oligomer-induced neurotoxicity is not clear; however, disruption of the following cellular effects have been proposed: 1) disruption of the mitochondrial morphology (Poehler *et al.* 2014; Chinta *et al.* 2010; Luth *et al.* 2014; Lindstrom *et al.* 2017); 2) increased endoplasmic reticulum stress (Colla *et al.* 2012b; Colla *et al.* 2012a; Castillo-Carranza *et al.* 2012); 3) inhibition of the ubiquitin-proteasome system (Lindersson *et al.* 2004; Emmanouilidou *et al.* 2010); 4) acceleration of membrane damage (Caughey & Lansbury 2003; Danzer *et al.* 2007); and 5) synaptic dysfunction (Choi *et al.* 2013; Schulz-Schaeffer 2010). The putative view that oligomers both precede the formation of, and are more cytotoxic than, Lewy bodies raises the question of why Lewy bodies are abundant in end-stage *post-mortem* brain tissue as oligomers would likely induce cell death prior to Lewy body formation. However, along with on-pathway prefibrillar oligomers, which eventually lead to mature fibrils, off-pathway stable oligomers might contribute to sustained toxicity (Lee *et al.* 2018). Furthermore, it is possible that Lewy body-bearing neurons are cells that have survived the initial oligomer-mediated cellular insult, and that they therefore should be viewed as markers of resilient neurons.

α-Synuclein as a target for diagnosis

Current diagnoses of synucleinopathies are based upon the constellation of clinical features, often coupled with supportive neuroimaging. However, symptoms in PD are only apparent when approximately 50-70% of nigral neurons are lost, implying that substantial neurodegeneration occurs before the disease is clinically detectable (Ross & Pickart 2004; Dauer & Przedborski 2003). DLB and MSA are thought to be substantially under-diagnosed (Kane *et al.* 2018; Joutsa *et al.* 2014), suggesting that current diagnostic methods are not sensitive enough to identify these disorders. Therefore, the development of biomarkers that can enable rapid and early differential diagnosis is a healthcare priority.

Among biochemical biomarkers, α-syn has been extensively studied as a candidate biomarker for PD in biological fluids especially in serum, plasma and cerebrospinal fluid (CSF) (Aasly et al., 2014; Gorostidi et al., 2012; Kasai et al., 2014; Simonsen et al., 2016; Landeck et al., 2016; Majbour et al., 2017; Mollenhauer et al., 2017). Some studies have reported decreased levels of total α-syn (t-α-syn) in CSF from patients with PD compared to control (Tokuda *et al.* 2006; Mollenhauer *et al.* 2008; Hong *et al.* 2010; Parnetti *et al.* 2011; Mollenhauer *et al.* 2011). However, measuring levels of t-α-syn alone does not distinguish

patients with PD from other synucleinopathies and there is a considerable overlap between patient and control groups (Mollenhauer *et al.* 2011). Soluble α-syn oligomers are elevated in brain homogenates from PD and DLB compared to control and, therefore, detection of α-syn oligomers in CSF and/or blood has been proposed as a potential biomarker (Paleologou *et al.* 2009; Sharon *et al.* 2003; Tokuda *et al.* 2010; El-Agnaf *et al.* 2006). Furthermore, the sensitivity and specificity of oligomeric α-syn is increased when it is calculated relative to t-α-syn (Tokuda *et al.* 2010; Parnetti *et al.* 2014; Hansson *et al.* 2014; Aasly *et al.* 2014; Majbour *et al.* 2016a; Majbour *et al.* 2016b). More recently, α-syn phosphorylated at S129 (p-S129-α-syn) has been proposed as a potential biomarker for PD and related disorders (Wang *et al.* 2012; Majbour *et al.* 2016b; Karampetsou et al., 2017). P-S129-α-syn is increased in PD CSF and, as with oligomeric α-syn, calculating the ratio of both α-syn oligomers/ t-α-syn as well as the ratio of p-S129-α-syn/t-α-syn increased the discriminating power between PD and healthy controls (Majbour *et al.* 2016b). Taken together, these studies indicate that identification and quantification of distinct α-syn proteoforms may have greater discriminative power than t-α-syn in identifying disease cases. However, the increased sensitivity conferred by evaluating such proteoforms relative to t-α-syn may suggest that synucleinopathies are characterized by dyshomeostasis of distinct α-syn proteoforms, rather than simple elevations in one particular sub-type.

Importance of antibodies in research/ diagnosis/ treatment

Antibodies (also known as immunoglobulins) are large Y-shaped proteins that are found in the blood or other body fluids of vertebrates. Antibodies are the key element of the adaptive immune system and recognize a unique part of foreign targets called antigens. Antibodies have several classes, of which immunoglobulin G (IgG) is most commonly used in biological studies. IgG antibodies are made and secreted by B cells and have the ability to recognize specific antigens. The IgG is made up of two subunits including two heavy chains and two light chains, assembled in a symmetrical structure. Each IgG has two identical antigen recognition domains, a Fab region and a constant stem region (Fc) that is common among the various classes of antibodies and is not involved in antigen recognition. Antibodies can be divided into monoclonal antibodies, that bind a single epitope, and polyclonal antibodies that recognize multiple epitopes on the same antigen.

Antibodies are commonly used to localize a specific antigen in tissue (immunohistochemistry), to quantitate the antigen by radioimmuoassay (RIA) or an enzyme-linked immunosorbent assay (ELISA), as affinity reagents in protein purification, to detect an antigen in tissue samples (western blotting), to concentrate an antigen (immunoprecipitation), and to neutralize an antigen. Due to the high binding specificity with the target antigen, and the ability to quantitate the amount of antigen within a sample using antibody-based assays, antibodies are widely used for diagnostics, fundamental studies probing disease mechanisms and, increasingly, therapeutics.

α-Syn specific antibodies: invaluable tools for synucleinopathy research

Several antibodies against α-syn have been developed (listed in Table 1) since it was first detected as the major component of LBs and Papp-Lantos bodies/GCIs (Spillantini *et al.* 1997; Spillantini *et al.* 1998b). α-Syn along with β-syn was identified in cytosolic extracts of the human brain using immunoblots with antibodies against phosphorylated tau proteins (Jakes *et al.* 1994). Subsequently, antibodies generated against purified Lewy bodies (Baba *et al.* 1998), the NAC region (amino acids 65-90), the N-terminal region and C-terminal region strongly label Lewy bodies (Culvenor *et al.* 1999; Dickson *et al.* 1999). However, antibodies raised against native α-syn likely label all or most α-syn within the brain, including the endogenous protein found in non-diseased brain, and may thus lack disease-specificity. The typically poor relationship between α-syn immunoreactivity, as identified using pan-α-syn antibodies, and important clinical variables such as phenotypic severity may be due to the fact that these antibodies can not distinguish changes in α-syn proteoform homeostasis, particularly proteoforms (oligomers/protofibrils) of putative disease importance.

In order to discriminate α-syn conformational forms, attempts were also made to generate single chain antibodies (scFvs) using the human phage antibody library (Maguire-Zeiss *et al.* 2006; Emadi *et al.* 2007; Nannenga *et al.* 2008). The localization of α-syn in neuronal nuclei in the brain was identified using antibodies raised against the full-length α-syn with an epitope mapped at the C-terminal region (amino acids 115-121) (Yu *et al.* 2007). The modified form of α-syn, including *in vitro* oxidized/nitrated forms, were also used to generate antibodies, showing extensive underappreciated α-syn pathology in the brains of diseased patients (Duda *et al.* 2002; Waxman *et al.* 2008). Aggregated α-syn, formed by treatment with either high salt or acid conditions, generated antibodies with high reactivity to disease associated α-syn (Mougenot *et al.* 2010; Kovacs *et al.* 2012). Owing to the role of α-

syn oligomers in the pathogenesis of synucleinopathies, modified oligomeric forms of α-syn induced by 4-hydroxy-2-noneal, have also been employed for generating antibodies (Fagerqvist *et al.* 2013). More recently, approaches have been developed either to generate either conformation-specific antibodies (Brannstrom *et al.* 2014; Covell *et al.* 2017) or antibodies targeting specific epitope within aggregated proteins using complementary peptide designs and grafting procedures (Sormanni *et al.* 2015).

Recently we have developed conformation-specific monoclonal antibodies that specifically recognize α-syn oligomers/protofibrils or fibrils with high affinity, without cross-reacting with monomers (Vaikath *et al.* 2015). The ability of these antibodies to specifically recognize aggregates of α-syn and not monomers or fibrils generated from other amyloidogenic proteins, makes them unique since most antibodies with conformational epitopes will cross-react with other proteins with similar conformations (Vaikath *et al.* 2015). Cross reactivity with proteins of similar conformations is a major problem in neurodegenerative disease research as the aged brain is characterized by many protein aggregates, many of which adopt similar conformations in pathological conditions (Attems & Jellinger 2013). These conformation-specific antibodies were able to label distinct burdens of Lewy pathology (Fig. 2). Furthermore, the conformational specificity of these antibodies have been utilized to detect α-syn aggregates in biochemical studies (Leung *et al.* 2015), in neurons of *C. elegans* (Kim *et al.* 2016) and rats (Duffy *et al.* 2018), guinea-pig ileum (Sharrad *et al.* 2017) human skin biopsy of patients with idiopathic PD (Donadio *et al.* 2018) and in human *post mortem* brain tissues from PD, DLB, AD and control cases (Vaikath *et al.* 2018)

Engineering Antibody fragments

Although antibodies are considered the "gold standard" in biomedical research and biofluid-based diagnostic assays, their therapeutic potential is undermined by their high molecular weight. This is a particular problem for neurological disorders as entrance of antibodies into the brain is inhibited by the blood-brain-barrier (BBB). Therefore, researchers have exploited the modular nature of antibodies to engineer customized antibody fragments without hindering their affinity or specificity. Recombinant antibody fragments including monovalent antibody fragments (Fab and scFv) and engineered variants, including diabodies, triabodies, minibodies and single-domain antibodies, are now emerging as replacements for full-length antibodies. In therapeutics, these fragments have higher penetrability in the tissues, including the brain, and faster clearance. They can be used to build multi-specific

antibody formats with high avidities. Moreover, their production in large quantities uses bacterial or yeast systems, and thus high quantities can be made in a more efficient and economic manner. The best-known form is scFv whose VH and VL domains are linked by a flexible polypeptide preventing their dissociation (Bird *et al.* 1988).

Another option is represented by the use of bifunctional antibodies. Recently, it has been demonstrated that amyloid-β:transferrin receptor antibodies have a high BBB penetrance and can be applied for efficient PET imaging and immunotherapy on an Alzheimer's disease mouse model (Sehlin *et al.* 2016; Syvanen *et al.* 2018). A similar strategy could possibly be applied towards α-syn for improved diagnostic and therapeutic strategies in synucleinopathies.

Antibody fragments targeting α-syn

In recent years, various antibody fragments have been generated to target α-syn. Zhou et al developed scFv fragments that bind and stabilize monomeric α-syn and thus prevent the formation of intracellular high molecular weight species (Zhou *et al.* 2004). scFv were also utilized to develop sensitive phage based capture ELISA to detect the protein from clinical samples with femtomolar range (Williams *et al.* 2015). Barkhordarain et al. (2006) combined the capabilities of phage display antibody technology and atomic force microscopy to isolate scFv antibody fragments that bind to a specific morphology of α-syn from synthetic libraries (Barkhordarian *et al.* 2006). Furthermore, scFvs have been isolated from a naïve human scFv library that was found to inhibit the aggregation of various amyloid proteins, and attenuate amyloid oligomer-induced cytotoxicity *in vitro* (Wang et al. 2009). These scFv recognized amyloid oligomers in all types of plaques, Lewy bodies, and amylin deposits in the brain tissues of AD and PD patients and in the pancreas of type 2 diabetes patients (Wang *et al.* 2009). Other scFv antibodies, including 10H and D5, were found to recognize morphologically distinct α-syn oligomers and selectively bind to aggregates in *post-mortem* human PD brain tissues and inhibit toxicity in a cell model (Emadi *et al.* 2007; Emadi *et al.* 2009) Recently, scFv W20 that recognizes various oligomers targeting the common epitopes of amyloid oligomers was found to reduce α-syn and mutant huntingtin protein aggregate load in mouse models, leading to reduced synaptic degeneration, neuroinflammation and oxidative stress, concomitant with amelioration of cognitive and motor deficits (Zha *et al.* 2016). Spencer *et al.* (2014) fused the scFv fragment, targeting oligomeric α-syn, to low density lipoprotein receptor-binding domain from apolipoprotein B, improving its therapeutic

potential by increasing accumulation of scFv in the brain and directing scFv/α-syn complexes for degradation through the endosomal sorting pathway (Spencer *et al.* 2014). They demonstrated that scFv directed against oligomeric α-syn (clone CD5-D5) expressed peripherally with lentiviral vectors (LV-CD5-D5-apoB) penetrates into the CNS and reduces the accumulation of wild-type α-syn, but not mutated α-syn associated with familial PD, *in vivo* (Spencer *et al.* 2014). Although nanobodies were found to be effective, their intracellular solubility can be low, Joshi et al (2012) demonstrated that fusing scFv intrabody with the proteasome-targeting PEST motif increased solubility whilst apparently also improving the functionality of the nanobody (Joshi *et al.* 2012).

Nanobodies targeting α-syn

A potential alternative to scFV antibodies are single domain antibodies, sometimes termed nanobodies, occurring in Camelidae (Hamers-Casterman *et al.* 1993). Like scFvs, nanobodies have high solubility and, with a molecular weight of 15 kDa, are capable of traversing the blood-brain-barrier. Nanobodies also bind antigens in the sub-nanomolar range and can be efficiently cloned in bacteria or yeast at relatively low cost. Among the first nanobodies generated against α-syn was NbSyn2 (Vuchelen *et al.* 2009) recognizing the C-terminus of human α-syn. Using a range of biophysical techniques, the epitopes of NbSyn2 and another nanobody, NbSyn87, were demonstrated to still be accessible when α-syn converts into its fibrillar structure, meaning they can label α-syn fibrils at different maturation stages (Guilliams *et al.* 2013). From a therapeutic perspective, NbSyn2 and NbSyn87 reduce oligomer-induced cellular toxicity *in vitro*, with important implications for future therapeutics (Iljina *et al.* 2017).

Therapeutic strategies in synucleinopathies

In addition to the continued use of antibodies for research and diagnosis, they are also currently being evaluated as potential therapeutic agents due to their high specificity, high binding affinity, long half lives and low toxicity. There is no disease-modifying therapy for any synucleinopathy but previous approaches have focused on reducing α-syn expression using antisense oligonucleotides (Murphy *et al.* 2000), miRNA (Junn *et al.* 2009) or siRNA (Spencer et al., 2019), decreasing α-syn aggregation with small molecules (Ardah et al., 2014; Ardah et al., 2015; Paleologou et al., 2005; Wrasidlo *et al.* 2016), increasing the

clearance of α-syn via autophagy (Sarkar *et al.* 2016) and preventing the seeding and prion like spreading of α-syn (Valera *et al.* 2016).

Currently, immunotherapeutic approaches are being explored as a potential disease-modifying treatment for synucleinopathies. Both active and passive immunotherapeutic approaches are being tested as a treatment strategy for various synucleinopathies. Recently, active immunotherapy, using PD01 (AFFiRiS AG), is reported to be under Phase 1 clinical trials (Valera & Masliah 2013). PD01 is a peptide-based vaccine that was found to reduce cerebral α-syn levels and ameliorate neuropathological alterations in mouse models of synucleinopathies (Schneeberger *et al.* 2012; Valera & Masliah 2013). Passive immunotherapy using antibodies against α-syn has been shown also to protect against neurodegeneration and reduce α-syn accumulation by triggering clearance via autophagy (Mandler *et al.* 2014; Masliah *et al.* 2011; Masliah *et al.* 2005). Likewise, monoclonal antibodies targeting NAC and C-terminal of α-syn ameliorated behavioral deficits, reduced nuerodegeneration and α-syn accumulation in neurons (Masliah *et al.* 2011) and glial cells (Bae *et al.* 2012). Immunotherapeutic approaches targeting the amyloid-β peptide have been the subject of several recent high-profile failures in phase III clinical trials in AD, prompting the suggestion that the wrong species of amyloid-β was engaged (Panza et al, 2019). In LB disease research, there are a paucity of studies fully characterizing the diversity and complexity of α-syn aggregates in the diseased brain with the aim of understanding those of greatest pathogenic relevance. As a previous study reported that antibodies generated against synthetic forms of α-syn characterized by high molecular weight species have little predictive and, presumably, therapeutic value (Woerman et al, 2018), future approaches may better approach this question by focusing on lower molecular weight species or isolated species from *post-mortem* brain tissue. Most previous studies have employed antibodies targeting monomeric or fibrillar α-syn. The detection of α-syn oligomers in the extracellular space (El-Agnaf *et al.* 2006; Tokuda *et al.* 2010; Lee *et al.* 2006) and its cell-to-cell propagation (Volpicelli-Daley *et al.* 2014; Danzer *et al.* 2012; Danzer *et al.* 2011; Luk *et al.* 2012; Helwig et al., 2016) makes it an ideal candidate target for immunotherapy. In a previous study, it was demonstrated that antibodies with strong selectivity towards α-syn oligomers/protofibrils could decrease α-syn pathology both in a cell model for α-syn aggregation and in the CNS of transgenic mice expressing the A30P variant of human α-syn (Nasstrom *et al.* 2011; Lindstrom *et al.* 2014). Furthermore, we have demonstrated that immunization with conformation-specific monoclonal antibodies reduces α-syn accumulation and related deficits in wild type α-syn transgenic (line 61) mice (El-Agnaf *et al.* 2017).

Moreover, the antibodies were most effective at reducing accumulation of α-syn oligomers in multiple brain regions and at preventing neurodegeneration (El-Agnaf *et al*. 2017). Therefore, immunotherapeutic targeting of α-syn oligomers holds considerable promise in the continued search for disease-modifying therapies for α-synucleinopathies.

There remains a legitimate question as to the timing of immunotherapeutic strategies in neurodegenerative proteopathies, with the criticism of such approaches being employed too late in the natural history of disease frequently used as *post-hoc* explanations of failure. It seems likely that immunotherapeutics will be most efficacious if applied early in the disease course, thus further underscoring the necessity of developing effective biomarkers to support early accurate diagnosis.

Conclusion

Relatively little is currently understood about how the protein α-syn contributes to the clinical and pathological features that characterize synucleinopathies. Many studies have evaluated α-syn using pan-α-syn antibodies that do not distinguish between proteoforms of likely differing clinical relevance, which may account for inconsistent findings tying α-syn expression with important clinical and pathological variables. It is evident that the knowledge base on the diversity of α-syn proteoforms and their clinical and pathological relevance is incomplete, and thus further studies are warranted to identify novel α-syn proteforms that may be potentially useful diagnostic and therapeutic targets. It is also likely that different strains of α-syn have different seeding capacities and thus the relative abundance of distinct species may be predictive of the rate of pathological propagation and, therefore, speed of functional decline. We suggest that there is a pressing need for further characterization of the diversity and clinico-pathological relevance of distinct strains of α-syn in LB disease. Antibody-based approaches across the translational spectrum, from discovery science to therapeutic targeting, are an excellent platform to accelerate discovery in this area.

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Acknowledgments and conflicts of interest disclosure

Dr. El-Agnaf laboratory is supported start-up funding from QBRI, HBKU, Qatar Foundation, Doha, Qatar. Brain tissue for images was provided from Newcastle Brain Tissue Resource, which is funded in part by a grant from the UK Medical Research Council, by NIHR Newcastle Biomedical Research Centre awarded to the Newcastle upon Tyne NHS Foundation Trust and Newcastle University, and by a grant from the Alzheimer's Society and Alzheimer's Research UK as part of the Brains for Dementia Research Project.

The authors declare that they have no Conflicts of Interest.

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Figure Legends

Figure. 1

α-Syn under physiological and pathological conditions.

Under physiological conditions, α-syn is an unstructured soluble monomer bound to membranes with two α-helices. Under pathological conditions, α-syn dimerizes and subsequently aggregates into oligomers/protofibrils, which ultimately form into mature fibrils.

Figure. 2

Conformation-specific antibodies label distinct burdens of Lewy pathology. Staining of the cingulate gyrus, a region prone to Lewy body deposition, in serial sections from a DLB case reveals distinct patterns of immunoreactivity. Antibodies against oligomeric α-syn (Syn-O1 and Syn-O2) label numerous fine neuritic structures in addition to larger Lewy bodies, whilst antibodies against fibrillar species (Syn-F1 and Syn-F2) label fine dots and Lewy bodies. Scale bars = 50 μm.

Table1. List of α-syn antibodies

Sl No	Antibody	Clonality	Host	Form	Antigen	Specificity	Tested application	Ref
1	LB509	mAb	Mouse	Culture supernatant	LB purified from DLB cortices	Generic	IHC, Western blot	Baba et al., 1998
2	Syn202	mAb	Mouse	Purified	Recombinant full length α-syn	Generic		Giasson et al., 2000
3	Syn204							
4	Syn205							
5	Syn208							
6	Syn211							
7	Syn214							
8	SNL-1	pAb	Rabbit	Purified	Recombinant full length α-syn	Generic	IHC, Western blot	
9	SNL-4	pAb	Rabbit	Purified	Recombinant full length α-syn	Generic	IHC, Western blot	
10	PER1	pAb	Rabit	Antiserum	amino acid 11-34		Immunoelectron microscopy	Crowther et al., 2000
11	PER4	pAb	Rabit	Antiserum	Recombinant full length α-syn	Generic		
12	H3C	mAb	Mouse	Purified	129-143 of Zebra Finch			
13	nSyn8	mAb	Mouse	Purified	nitrated α-syn	Generic	ELISA, IHC, Western blot	Giasson et al b., 2000
14	nSyn14							
15	nSyn24							
16	Anti-3NT	pAb	Rabbit	Purified	nitrated α-syn	Generic		

17	Syn 303	mAb	Mouse	Purified	Oxidized α-syn	Generic	IHC, Western blot	Duda et al., 2002; Waxman et al ., 2008
18	Syn 505	mAb	Mouse	Purified				
19	Syn 514	mAb	Mouse	Purified				
20	Syn1	mAb	Mouse	Purified	rat α-syn	Generic	Western blot	Perrin et al., 2003
21	FILA-1	pAb	Rabbit	Purified	Sucrose density gradient-purified α-syn filaments	Generic	Dot blot, Solid phase binding assay	Lindersson et al., 2004
22	15 G7	mAb	Mouse	Purified	Synthetic peptide	Generic	IHC	Croisser et al., 2006
23	42	mAb	Mouse	Purified	Rat synuclein amino-acid 15-123	Generic		
24	7B 2, 12	mAb	Mouse	Purified	Recombinant full length α-syn	Generic		
25	KM51	mAb	Mouse	Purified	Recombinant full length α-syn	Generic		
26	NAC1	scFv	Yeast display library	Purified	non-immune human scFv library	Generic	Western Blot	Lynch et al., 2008
27	NAC3							
28	NAC6							
29	NAC14							
30	NAC24							
31	NAC32							
32	Panel of anibodies	pAb	Rabbit	Purified	α-syn peptide corresponding to different regions	Generic	ELISA, Dot blot	Masuda et al., 2009
33	NbSyn2	Nanobody	NA	Purified	Recombinant full length α-syn	Generic		Vuchelen et al., 2009; De

								Genst et al., 2010
34	AS11	mAb	Mouse	Purified	Modified α-syn	Generic	Western blot, IHC, immunofluorescence	Mougenot et al., 2010
35	2 E 3	mAbs	Mouse	ND	α-syn recombinant protein	Generic	IHC	Vivacqua et al., 2011
36	2 D 5							
37	9 E 4	mAb	Mouse	ND	α-syn recombinant protein			Masliah et al., 2011
38	mAb49/G	mAb	Mouse	Purified	HNE-induced α-syn oligomers	Conformation-specific	ELISA, Immunocytochemistry	Nasstrom et al., 2011
39	5G4	mAb	Mouse	Purified	α-syn peptide corresponding to amino acids 44-57	Generic	ELISA, Immunocytochemistry	Kovacs et al., 2012
40	mAb38F	mAbs	Mouse	Purified	HNE-induced α-syn oligomers	Conformation-specific	ELISA, Immunocytochemistry	Fagerqvist et al., 2013
41	mAb38E2							
42	Syn-F1	mAbs	Mouse	Purified	Recombinant full length α-syn	Conformation-specific	Dot blot, ELISA, IHC	Vaikath et al., 2015
43	Syn-F2							
44	Syn-O1							
45	Syn-O2							
46	Syn-O3							
47	Syn-O4							
48	11D12	mAb	Mouse	Purified	Recombinant full length α-syn	Generic	Dot Blot, Western blot	Majbour et al., 2016b

49	PS129	mAb	Mouse	Purified	α-syn peptide corresponding to amino acids 125-123 phosphorylated at S129	Phospho-speicfic	Dot Blot, Western blot	
50	Syn-140	pAb	Sheep	Purified	Recombinant full length α-syn	Generic	Western blot	
51	A1-A6	mAbs	Mouse	Purified	Recombinant full length α-syn monomer-aggregate mixture	Generic	Dot blot	Sahin et al., 2017
52	Syn7015	mAbs	Mouse	ND	sonicated, cross-linked α-syn stain A PFFs	Conformation-specific	IHC	Covell et al., 2017
53	Syn9029							
54	A panel of antibodies	mAbs	Mouse	ND	α-syn peptide corresponding to N and C-terminal	Generic	Western blot, IHC	Dhillon et al., 2017

**ND: mAb: monoclonal antiobdy; pAb: Polyclonal antibody; ND: Not defined; WB: Western blot; IHC: Immununohistochemistry;*

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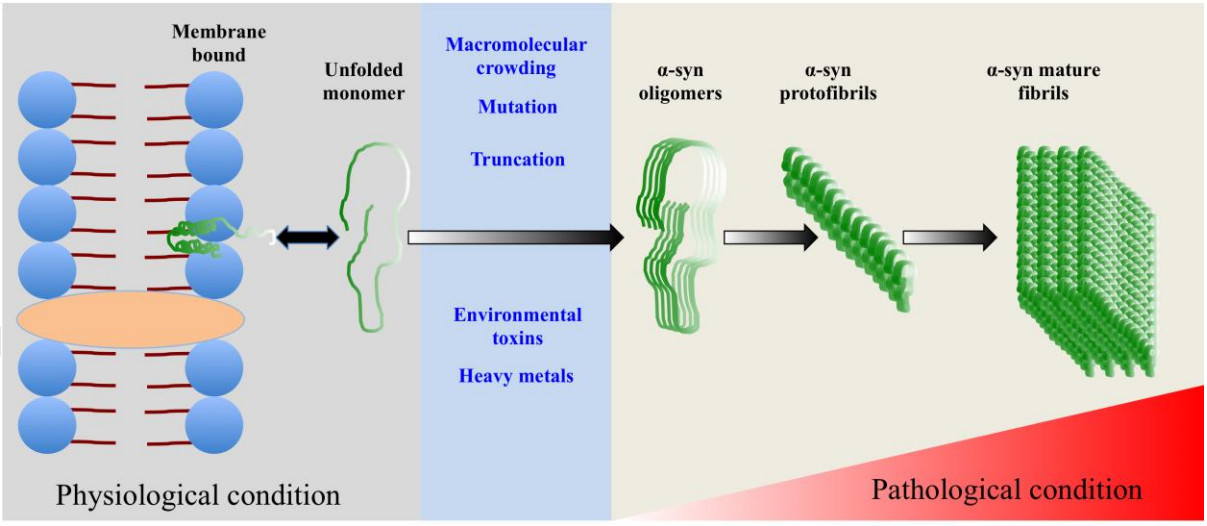

Figure 1