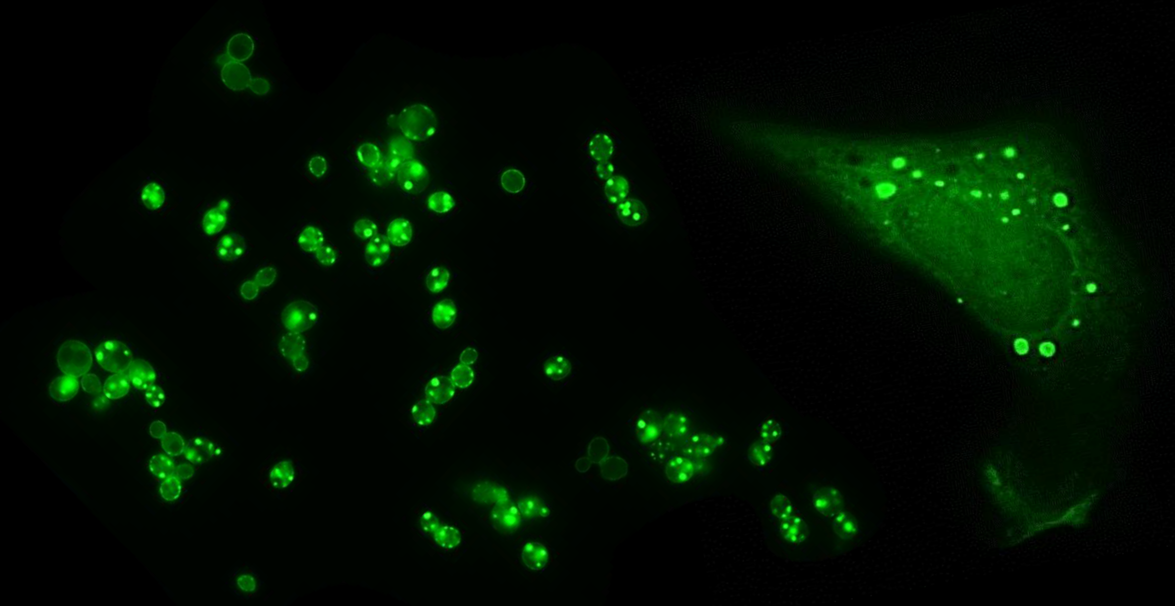
Investigating the neuroprotective effect of plant (poly)phenols in models of Parkinson's disease

Diana L. Constantino Macedo



Dissertation presented to obtain the Ph.D. degree in:

Biochemistry, specialization in Neurosciences
Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

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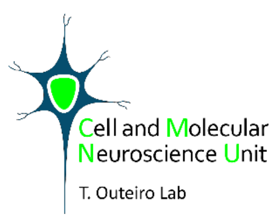

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Thank you!

Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disease, and affects 1-2% of the population worldwide after the age of 65 years. Currently, there are only symptomatic treatment for PD and these do not mitigate the underlying causes of the disease.

There is strong hope that the deeper we scrutinize the pathobiology of PD, the closer we will become to identifying useful drug targets and design an effective pleiotropic drug. This drug should be able to act in the multitude of events leading to disease, ultimately fighting neurodegeneration.

PD is intimately associated with the misfolding and aggregation of alpha-synuclein (aSyn), a protein primarily found in pre-synaptic neurons. aSyn is found aggregated in cytosolic inclusions known as Lewy bodies (LBs), the main pathological hallmark of PD and other synucleinopathies.

Strikingly, 90% of aSyn appears to be phosphorylated on serine 129 (pS129) in LBs of patients with synucleinopathies. Nevertheless, the normal and pathological function of this post-translational modification is still unresolved. The present study analysed the role of pS129 in a simple but versatile yeast model of PD, by using a phospho-resistant aSyn form, S129A. Blocking aSyn phosphorylation on S129 increased aSyn toxicity and promoted the formation of cytosolic inclusions. Furthermore, unphosphorylated aSyn exacerbated vesicular trafficking defects and failed to induce autophagy, leading to the accumulation of aSyn.

Autophagy is the main pathway by which large protein aggregates are degraded and cleared from the cell. Modulations of phosphorylation on S129 and of autophagy are considered potential drug targets in PD. However, in addition to the identification of drug targets, it is urgent to develop novel therapeutic strategies, and to identify molecules able to modify the cellular mechanisms involved in neurodegeneration. Thus, we further explored PD pathways using the yeast model of PD to study the potential of (poly)phenols as neuroprotectants.

The ubiquity of (poly)phenols in fruits and vegetables, allied with epidemiological/research studies reporting their ability to reverse and prevent age-related cognitive decline, made (poly)phenols tempting subjects of study in PD.

(Poly)phenols occur naturally in plants, which constitute a valuable source of these compounds to be explored in pharmacological or nutritional approaches. In this thesis, we explored Portuguese plant species to study the potential of (poly)phenols in the pathobiology of PD.

In an initial pharmacological/nutraceutical perspective, (poly)phenols were extracted and identified from leaves or fruits from the species *C. album*, *A. unedo*, *G. biloba* and *R. idaeus*. Interestingly (poly)phenols from the leaves of *C. album* presented the strongest properties in phenotypic assays of cell viability, metabolism, and aSyn aggregation.

The mechanism of action underlying the protection provided by these (poly)phenols was found to be related with the inhibition of aSyn fibrillization and stabilization of oligomeric species *in vitro* and, remarkably, these species were not toxic in a human cell model of PD. Furthermore, (poly)phenols reduced oxidative stress, the formation of aSyn inclusions in yeast, and likely promoted the clearance of aSyn through the promotion of autophagy, both in yeast and in human H4 cell models of PD.

To address the nutritional role of (poly)phenols, an *in vitro* digestion procedure that mimics the upper gastro-intestinal digestion was used. In a previous study, we reported the effect of digestion in the chemical profile of fruits and leaves from *A. unedo* and their ability to protect yeast cells from an oxidative insult. Thus, metabolites from *A. unedo* were analysed in the yeast model of PD. The digested metabolites from the leaves of *A. unedo* were protective against aSyn toxicity, and reduced the formation and size of aSyn inclusions. Importantly, it regulated pathways intimately associated with protein misfolding, including oxidative stress, mitochondrial dysfunction, ER-stress and autophagy and proteasomal function.

In conclusion, a better understanding of the molecular mechanism underlying PD, in particular the role of S129 aSyn phosphorylation, opens novel avenues for the identification of new drug targets and the development of more effective therapies. (Poly)phenols proved to be a promising source of therapeutic agents for neurodegenerative diseases, oxidative stress or unfolded protein disorders, as a pharmacological/nutraceutical or nutritional approach.

Resumo

A doença de Parkinson (DP) é a segunda doença neurodegenerativa mais comum e afecta 1-2% da população mundial com idade superior a 65 anos. Atualmente apenas existem tratamentos sintomáticos para a DP e estes não mitigam as causas subjacentes da doença.

Existe grande espectativa de que quanto mais profundamente estudarmos a patobiologia da DP, mais nos aproximemos da identificação de alvos terapêuticos úteis e de desenhar um fármaco pleiotrópico eficaz. Este fármaco deve ser capaz de agir na multiplicidade de eventos que levam à doença, combatendo a neurodegeneração.

A DP está intimamente associada com a conformação incorreta e a agregação de alfa-sinucleína (aSyn), uma proteína principalmente presente em neurónios pré-sinápticos. aSyn é encontrada agregada em inclusões citosólicas conhecidas por corpos de Lewy, a principal característica patológica de DP e outras sinucleinopatias. Apesar da intensa pesquisa e do vasto conhecimento criados nos últimos anos, um claro exemplo de que a patobiologia da DP ainda representa um desafio vem da falta de compreensão sobre o papel da fosforilação na agregação da aSyn.

Surpreendentemente, 90% da aSyn parece ser fosforilada na serina 129 (fS129) nos corpos de Lewy em pacientes com sinucleinopatias. No entanto, a função normal e patológica desta modificação pós-transcripcional está por resolver. No presente estudo analisamos o papel da fS129 num modelo simples mas versátil de DP em levedura, usando uma aSyn fosfo-resistente, S129A. O bloqueio da fosforilação na S129 da aSyn aumentou a toxicidade da aSyn e promoveu a formação de inclusões citosólicas. Além disso, aSyn não fosforilada exacerbou o deficiente tráfico vesicular e não conseguiu induzir autofagia, levando à acumulação de aSyn.

A autofagia é a principal via pela qual grandes agregados proteicos são degradados e eliminados da célula. A modulação da fosforilação na S129 e da autofagia são considerados como potenciais alvos terapêuticos em DP. No entanto, além da identificação de alvos terapêuticos é urgente o desenvolvimento de novas estratégias terapêuticas, e a identificação de moléculas capazes de modificar os mecanismos celulares envolvidos na neurodegeneração. Assim, neste trabalho exploramos os mecanismos da

DP, usando a levedura como modelo, para estudar o potencial neuroprotector de (poli)fenóis.

A ubiquidade de (poli)fenóis em frutas e legumes, aliado aos estudos epidemiológicos/científicos relatando a sua capacidade de reverter e prevenir o declínio cognitivo relacionado com a idade, tornou os (poli)fenóis moléculas atrativas no estudo na DP.

Os (poli)fenóis ocorrem naturalmente em plantas, que constituem uma valiosa fonte destes compostos a ser analisada em abordagens farmacológicas ou nutricionais. Nesta tese, exploramos espécies de plantas portuguesas e estudamos o potencial dos seus (poli)fenóis na DP.

Numa inicial perspetiva farmacológica/nutracêutica, (poli)fenóis foram extraídos e identificados a partir de folhas ou frutos das espécies *C. album*, *A. unedo*, *G. biloba* e *R. idaeus*. Curiosamente, os (poli)fenóis das folhas de *C. album* apresentaram propriedades promissores em ensaios fenotípicos de viabilidade, metabolismo ou agregação de aSyn.

O mecanismo de ação subjacente à proteção oferecida por estes (poli)fenóis estava relacionado com a inibição da fibrilização da aSyn e estabilização de espécies oligoméricas *in vitro*. Notavelmente, estas espécies não exibiram toxicidade num modelo celular humano de DP. Adicionalmente, os (poli)fenóis reduziram o stresse oxidativo, as inclusões de aSyn em levedura, e estimularam a degradação de aSyn através da promoção de uma autofagia funcional, em modelos de PD em levedura e células humanas H4.

Para contemplar o papel nutricional dos (poli)fenóis estes foram submetidos a um procedimento de digestão *in vitro* que mimetiza a digestão gastrointestinal superior. Num estudo anterior, reportamos o efeito de digestão no perfil químico de frutos e folhas de *A. unedo* e a sua capacidade de proteger as células de levedura de um insulto oxidativo. Consequentemente, os metabolitos de *A. unedo* foram analisados no modelo de PD em levedura. Os metabolitos digeridos das folhas de *A. unedo* exibiram proteção contra a toxicidade da aSyn, reduziram a formação e o tamanho dos agregados de aSyn. É importante realçar que os (poli)fenóis regularam vias intimamente associadas com a mal-conformação proteica, incluindo stresse oxidativo, disfunção mitocondrial, stresse do reticulo endoplasmático e sistemas de controlo de qualidade, como autofagia e proteasoma.

Em conclusão, a melhor compreensão dos mecanismos moleculares da DP, em particular, o papel da fosforilação no resíduo S129 da aSyn, abre caminho para a identificação de novos alvos terapêuticos e para o desenvolvimento de terapias mais eficazes. Os (poli)fenóis revelaram-se uma promissora fonte de agentes terapêuticos em doenças neurodegenerativas, relacionadas com o stresse oxidativo ou agregados proteicos, como uma nova abordagem farmacológica/nutracêutica ou nutricional.

Thesis outline

This thesis is organized in five parts: introduction, three chapters of experimental work and a general discussion. In the general introduction the topics necessary for the broad comprehension of this thesis are described. It is briefly defined neurodegenerative diseases, PD pathology and its key players and pathways. Followed by information concerning yeast as a model for PD and how it can be used to screen for protective molecules. (Poly)phenols are also described as well as its pharmacological and nutritional importance, and the main findings regarding its neuroprotective properties. Finally, a short introduction to the plant species used in this study is made. Part of this section was included in a review published in *Microbial cell*.

The experimental work is divided in 3 chapters. The first chapter exploits a yeast model of PD to investigate the molecular mechanisms underlying the pathobiology of the disease. More specifically, we explored the role of aSyn phosphorylation in PD, and its effect on protein clearance. The work presented in this chapter was integrated in a publication in *PLoS Genetics*.

In the second chapter, a (poly)phenol enriched-fraction from *Corema album* leaves, who exhibited neuroprotective potential, was tested for protection against aSyn toxicity in yeast and H4 cell models of Parkinson's disease. Furthermore, its mechanisms of protection where explained, relying on a pharmacological/nutraceutical approach to (poly)phenols. This chapter was published in *Human Molecular Genetics.*

The third chapter is the continuation of a manuscript in preparation for publication (Jardim et al.), and comprises a nutritional approach to (poly)phenols. The (poly)phenols from *Arbutus unedo* leaves where submitted to an *in vitro* gastrointestinal process, subsequently its neuroprotective potential was determined and its protection mechanisms were analysed. From this work resulted a manuscript awaiting for peer review (Macedo et al.).

Finally an integrated discussion of the findings obtained is presented, as well as the main considerations and potential direction of future work.

Abbreviations list

AAPH: 2, 2' azobis (2-amidopropane) dihydrochloride

Aβ: β-amyloid

AD: Alzheimer's disease

APP: amyloid precursor protein

aSyn: alpha-synuclein

Atg8: autophagy-related protein 8

CSM: complete supplement mixture

DCF: 2',7'-dichlorfluorescein

DCFHDA: 2',7'-dichlorfluorescein-diacetate

DT: doubling time

ER: endoplasmatic reticulum

ERAD: endoplasmatic reticulum associated degradation

ERAA: endoplasmatic reticulum associated autophagy

GAE: gallic acid equivalents

iNOS: inducible nitric oxide synthase

MDR: multi-drug resistance

MFI: median fluorescence intensity

OD: optical density

ORAC: oxygen radical absorbance capacity

PD: Parkinson's disease

PDA: photo diode array

PDM: (poly)phenol-digested metabolites

PEFs: (poly)phenol-enriched fractions

PGK: phosphoglycerate kinase

PI: propidium iodide

ROS: reactive oxygen species

RNOS: reactive oxygen and nitrogen species

SC: synthetic complete

SD: standard deviation

Sirt1: sirtuin 1

SOD: superoxide dismutase

SPE: solid phase extraction

TCA: trichloroacetic acid

TE: trolox equivalents

ThT: thioflavin T

UPS: ubiquitin-proteasome system

YAP1: yes-associated protein

YPD: yeast extract, peptone and dextrose

Introduction

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1. Neurodegenerative diseases

Neurodegenerative diseases are a group of incurable and debilitating conditions that emerge from a progressive nervous system dysfunction, as a consequence of the loss of neuronal cells [1]. These diseases can arise from hereditary and sporadic conditions, they have a complex multifactorial pathogenesis and are often associated with atrophy of the central or peripheral structures of the nervous system [2]. Neurodegenerative diseases comprise disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD) and prion diseases.

Since neurodegenerative diseases strike primarily in mid- to late-life, their incidence is expected to rise due to the increase in life expectancy observed in the last century. In 2004, the WHO estimated that neurodegenerative diseases reached 29.4 million people worldwide, posing tremendous social and economic challenges to modern societies.

1.1. Synucleinopathies

Neurodegenerative diseases are highly heterogeneous pathologies, affecting different parts of the brain and having different etiologies. The diseases that rise from the misfolding or aggregation of proteins are collectively described protein conformational disorders. Proteins, as the main effectors in the cell, play underpinning roles in all biological processes. Therefore, as new proteins are identified and their functions understood, the list of protein conformational disorders expands incessantly.

The aggregation and toxicity of amyloidogenic proteins have been implicated in the pathobiology of disorders such as AD, HD, PD, and prion encephalopathies [2, 3]. The neurodegenerative diseases that share the aggregation of the protein α-synuclein (aSyn) as a primary feature are collectively known as synucleinopathies. These disorders include PD, dementia with Lewy bodies, pure autonomic failure, and multiple systems atrophy. Clinically, they are characterized by a chronic and progressive decline in motor, cognitive, behavioural, and autonomic functions, depending on the distribution of the pathognomonic lesions [1, 2].

aSyn is a ubiquitous and abundant brain protein that belongs to a family of three distinct proteins, aSyn, β-synuclein and γ-synuclein, that have only been found in vertebrates [4]. It is known that aSyn is a small protein comprising 140 amino acid residues, and is encoded by the *SNCA* gene. It is expressed at high levels in the brain and in neural synaptic terminals. However, although its function is still partially unclear, it is thought to be involved in the regulation of dopamine neurotransmission, vesicular trafficking and in synaptic function and plasticity [5, 6 4, 7, 8].

The synucleins were associated with neurodegenerative diseases after the initial report of an AD non-amyloid-β (Aβ) component, consisting of a 35 amino acid residue polypeptide generated by cleavage of aSyn[9]. A few years after, the *PARK1* locus, which encodes for aSyn, was associated to PD [10]. This discovery was then followed by a report identifying aSyn in Lewy bodies (LBs), described as concentric hyaline cytoplasmic inclusion bodies[11, 12]. All LBs were shown to contain the protein aSyn[13, 14]. After this initial discovery, this protein was detected in cellular inclusions in several other neurodegenerative diseases including dementia with LBs and multiple systems atrophy[4].

1.2. Parkinson’s disease

PD was first characterized by James Parkinson in 1817, who described this synucleinopathy as a loss of dopaminergic neurons in the *substantia nigra pars compacta*, accompanied by muscle rigidity, bradykinesia, resting tremor and postural instability[15].

LBs, the pathological hallmark of this disease, can be detected in the surviving dopaminergic neurons *via* histological analysis of the brains of patients. These cytoplasmic inclusions enclose the protein aSyn, as well as molecular chaperones, proteasomal and lysosomal subunits[12].

PD is one of the most common neurodegenerative diseases, with a prevalence of 1-2% in people over 65 years old and 4-5% in people over 85 [16]. European studies on the prevalence and incidence of PD showed that the ageing of the human population will lead to tremendous increase in the number of affected individuals[17]. In Portugal, a study on the prevalence of PD showed that age standardized rates are 1.4/1000 and 1.3/1000 for male and female genders, respectively. The rates increase consistently in both genders with age,

with yearly increments ranging from 0.6% in 65 year old patients to 3.5% in 85 or older. In Portugal, PD reaches 9/1000 in individuals with "75 or more" years[18].

Almost two centuries past after the first description of PD, we still lack an effective treatment for this illness. The drug discovery process for synucleinopathies has been delayed, in part, by its complex and multifactorial etiology, which poses a challenge to the understanding of the mechanisms leading to neurodegeneration. Thus, it is imperative to target research to investigate the genes and proteins involved in PD, both in terms of their normal physiological function and their role in this synucleinopathy [19].

1.2.1. Parkinson's disease pathobiology

PD symptoms arise from the loss of dopaminergic neurons and reduced levels of dopamine [20] (Fig. 1). Dopamine functions as a neurotransmitter in the brain. Dopamine plays a major role in the brain reward system and is involved in motor control. Its chemical structure comprises an amine that is formed by removing a carboxyl group from a molecule of L-DOPA [20].

The presence of unfolded, aggregated and ubiquitylated proteins in the LBs of dopaminergic neurons indicates that proteostasis dysfunction is a common theme underlying the different etiologies of PD [12, 21]. These alterations play a significant role in the initiation, development and/or progression of the neurodegenerative process in PD. Concomitantly, one of the pathways from which cell toxicity arises in PD is through aSyn misfolding and aggregation [22, 23]. The *SNCA* gene, encoding for aSyn, was the first loci associated with familiar and sporadic cases of PD [10]. Genetic alteration in this gene, including triplication[24] and duplication[25], as well as missense mutation A30P[26], E46K[27], H50Q[28], G15D[29], A53T[10] and A53E[30] are described as causing autosomal dominant forms of this disease. These mutations affect aSyn interaction with membranes and propensity to aggregate [31-34].

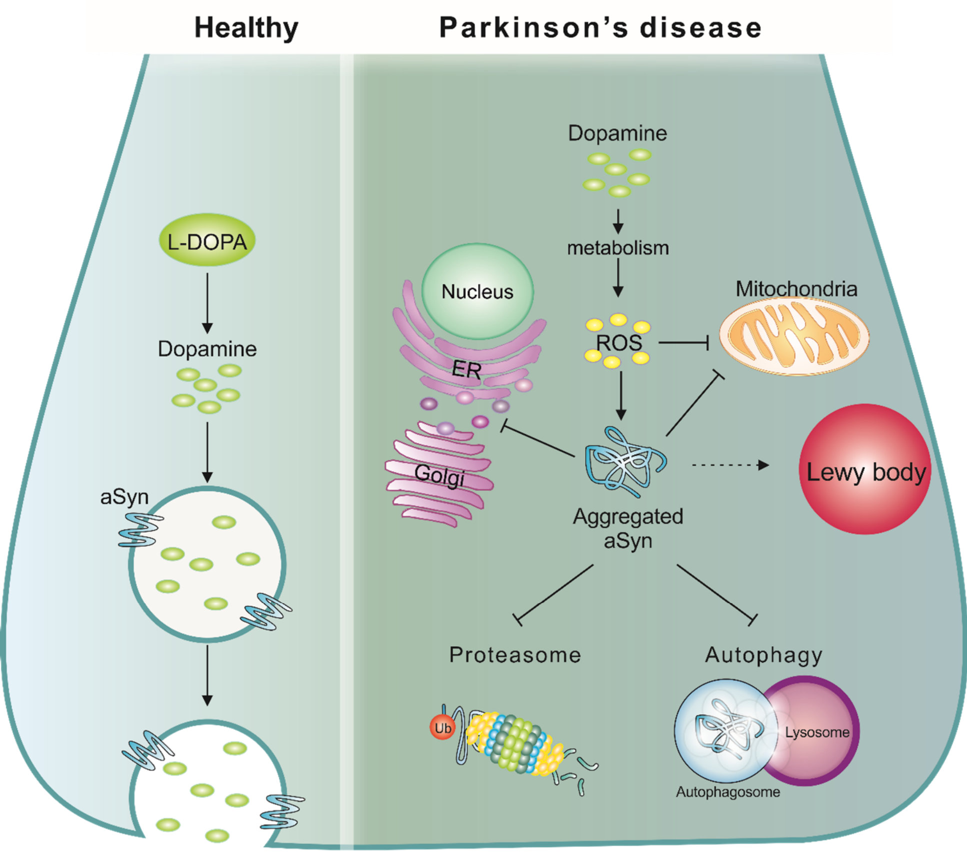


Figure 1: Parkinson's disease pathobiology. In a healthy neuron the neurotransmitter dopamine is transported to the synaptic terminal in a synaptic vesicle, which fuses to the membrane of the neuron and releases the neurotransmitter, resulting in signalling in the postsynaptic neuron. Alpha-synuclein (aSyn) is involved in the formation of the synaptic vesicle. aSyn in its functional form is bonded to membranes, although, pathological conditions results in aSyn aggregation and impaired vesicular binding, which abrogates vesicle trafficking. As a consequence in Parkinson's disease (PD) the vesicular transport is disrupted and dopamine is not transported or released, the lack of a neurotransmitter signalling leads to neurodegeneration. Endoplasmatic reticulum (ER)-to-Golgi vesicular transport is also impaired by aSyn aggregation. Oxidative stress caused by reactive oxygen species (ROS) formation, and dysfunctional mitochondrial metabolism is also responsible for cell toxicity in PD. Moreover, dopamine metabolism and impaired neurotransmitter release leads to the formation of ROS. To overcome aSyn-induced toxicity healthy cells have protein quality control systems to deal with misfolded and aggregated proteins. aSyn can be degraded mainly by the proteasome or autophagy. However, failure of protein clearance pathways and accumulation of misfolded proteins, mediated by aSyn and aging, leads to the formation of protein aggregates and LBs, ultimately resulting in neurodegeneration (adapted from Cell signalling).

Despites the clear relevance of aSyn in PD pathogenesis, several other genes linked to heritable, monogenic PD have also been described. Including the leucine-rich repeat kinase 2 LRRK2; the E3 ubiquitin-ligase Parkin; the mitochondrial PTEN-induced putative kinase 1 PINK1; the oxidation-sensitive chaperone DJ-1; and the lysosomal ATPase ATP13A2 [35]. Additionally, mutations in several genes are known to increase the risk of developing PD, standing out the vacuolar protein sorting 35 homolog VPS35; the ubiquitin carboxyl-terminal esterase L1 UCH-L1; the translation initiation factor 4-gamma 1 EIF4G1; and the beta-glucocerebrosidase (GBA)[35]. The public online database 'PDGene' (http://www.pdgene.org) provides a comprehensive list of PD genetic risk factors, recently identified in large-scale meta-analysis of genome-wide association studies [36, 37]. The proteins encoded by these genes are involved in processes such as synaptic vesicles [38, 39], autophagy [40, 41], neurite outgrowth [42], endocytosis [43], mitochondrial morphology [43] and function [44], mitophagy [45-50] and oxidative stress responses [51]. Overall, the genetics of PD highlights the multifactorial aspect of the disease, underscoring the importance of mitochondrial function, membrane trafficking and protein quality control systems in the pathophysiology of PD (Fig. 1).

aSyn aggregation is implicated in the loss of dopaminergic neurons and defects in dopamine transport. Soluble aSyn is a competitive inhibitor of tyrosine hydroxylase, the rate-limiting step in tyrosine to L-DOPA biosynthesis [52]. aSyn in its functional form is bonded to membranes. Although, pathological conditions results in aSyn impaired vesicular binding, which abrogates lipid-mediated signalling cascades and vesicle trafficking [19, 52]. Therefore, the equilibrium between lipid-associated (folded) and cytoplasmic (aggregated) aSyn seems to provide a mechanistic link between dopamine production, packaging and vesicle dynamics. Moreover, impaired neurotransmitter release might lead to the formation of reactive oxygen species (ROS) [53] (Fig. 1).

Oxidative stress and dysfunctional mitochondrial metabolism is also responsible for cell toxicity in PD [54]. There is evidence that a reduction in oxidative phosphorylation and a decrease in complex 1 activity, leading to ROS formation and oxidative stress, are related to PD [55]. In parallel, there is a loss in mitochondrial membrane potential, leading to the

release of cytochrome C from the intermembrane space to the cytosol, and activation of the intrinsic apoptotic pathway, resulting in caspase activation and cell death [56].

To overcome aSyn-induced toxicity cells have protein quality control systems to deal with misfolded and aggregated proteins. aSyn can be degraded by the ubiquitin-proteasome pathway (UPS), chaperone mediated autophagy (CMA) and macroautophagy [57]. However, failure of protein clearance pathways and accumulation of misfolded proteins with age leads to the formation of protein aggregates and cell toxicity [22] (Fig. 1).

The dysfunction in either of the pathways, protein clearance or mitochondrial pathway, leads to oxidative stress which causes malfunction of these very same pathways by feedback and feed-forward mechanisms, resulting in irreversible cell damage and death [19] (Fig. 1).

Despite the significant progress in understanding the molecular basis of neurodegeneration, the lack of known useful molecular targets for therapeutic intervention has slowed down the scientific discovery processes. Thus, combating PD by preventing or reversing the build-up of toxic protein aggregates, mainly composed of aSyn, may be an interesting therapeutic approach.

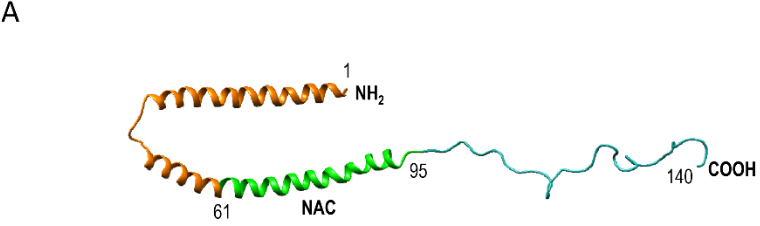
1.2.1.1. Alpha-synuclein misfolding and aggregation

Intense efforts have been made to characterize the sequence and structure of aSyn. This 14.5 kDa protein has an amphipathic lysine-rich terminus, an aggregation prone central region and a disordered acidic carboxy-terminal tail[58, 59] (Fig. 2).

The N-terminal region of aSyn contains four 11 residue imperfect repeats with a highly conserved hexameric motif (KTKEGV)[10, 60]. This region is responsible for aSyn interaction with membranes and the PD aSyn mutations were found in this terminal region[10, 60] (Fig. 2).

The central region of aSyn was first identified in AD amyloid plaques and was named non-amyloid component region (NAC), it is composed predominantly by hydrophobic residues[9]. The NAC region is necessary for aSyn aggregation, as shown by the deletion of segments within this motif [61, 62].

A



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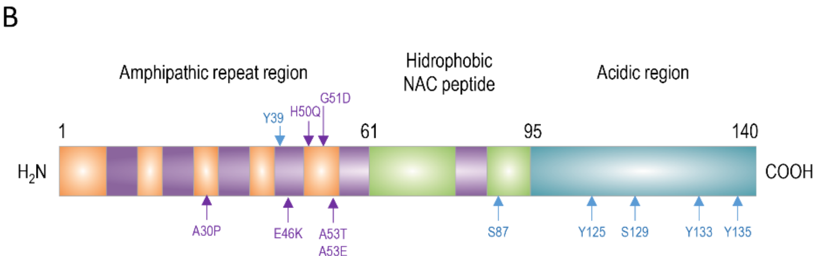

Figure 2: Biochemical structure and schematic representation of aSyn. A) Biochemical structure of micelle-bound aSyn (Protein Data Bank ID:1XQ8). The N-terminal region, the non-amyloid-β component of Alzheimer's disease amyloid plaques (NAC) region and the C-terminal part are coloured orange, green and blue, respectively. Numbers refer to amino acid residues flanking the different regions. **B)** Schematic representation of aSyn showing the aSyn residues that can be phosphorylated in blue and the mutations associated with familial PD are shown in purple. The N-terminal amphipathic region of the protein is represented in orange, the hydrophobic central region containing the NAC peptide is represented in green and the highly acidic C-terminal is represented in blue. The imperfect KTKEGV repeats are represented in purple.

The C-terminal region is highly enriched in acidic (glutamate and aspartate) and proline residues and is highly disordered [63, 64]. It has been suggested that this region plays a critical role in modulating the stability, structure, aggregation and function of aSyn *in vivo* [65]. Additionally, it has been implicated in the regulation of aSyn interactions with proteins [66, 67 68, 69], metals [70, 71] and small molecules (e.g. dopamine and polyamines) [72]. The C-terminal region also contains the majority of the known aSyn PTMs sites [60], including the phosphorylation sites (Fig. 2).

In solution, aSyn (WT, A30P, E46K and A53T) exists as an ensemble of disordered conformations [73]. The study of the familiar forms of PD contributed significantly to the understanding of PD intimate relation with aSyn misfolding and aggregation. The missense mutations that alter the N-terminus of aSyn, potentiating the formation of protofibrils (A30P)

or mature fibrils (A53T and E46K), and as well as genomic multiplications of the aSyn encoding gene, led to an increase in the cytoplasmic accumulation of the aSyn monomer and potentiated its aggregation and toxicity [74-76].

Several other factor also contribute to triggering the aggregation of aSyn, including post-translational modifications (PTMs) and interactions with specific metals [70, 71] and small molecules [72]. Fibril formation by aSyn proceeds through a series of discrete oligomeric intermediates, known as protofibrils of different sizes and morphologies, including spherical, annular and chain-like structures [13, 20] (Fig. 3).

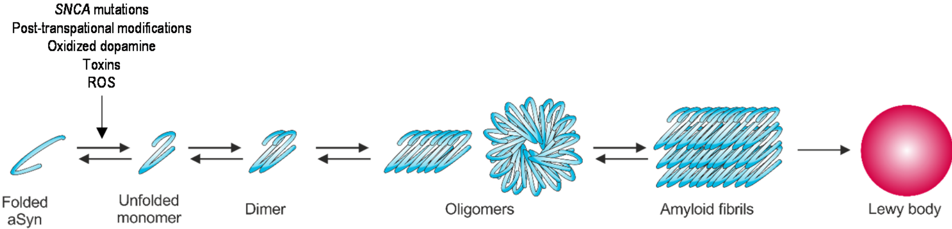

Figure 3: Putative pathway of aSyn aggregation. In the cytosol, aSyn can unfold due to *SNCA* mutations, PTMs, oxidized dopamine, reactive oxygen species (ROS) or toxins. This process can be reverted by chaperones. Unfolded aSyn monomers interact to form initially unstable dimers, which grow slowly to generate oligomers of varying morphologies, including transient spherical and ring-like oligomers that eventually originate fibrils. The aSyn oligomers are in equilibrium with monomers and convert to fibrils by monomer addition via a nucleated polymerization mechanism. The accumulation of these amyloid fibrils leads to the formation of intracellular inclusions called Lewy bodies.

The ‘amyloid hypothesis’ states that the aggregation of proteins into an ordered fibrillar structure is causally related to aberrant protein interactions that culminate in neuronal dysfunction and ultimately neurodegeneration[77].

Despite the well-known connection between protein misfolding, aggregation and disease, the way by which misfolding results in disease is not clearly understood. In the “gain of toxic function” theory proteins acquire toxic conformations, which may disrupt the homeostasis of cells, and the respective tissues and organs. In “the loss of function” theory the lack of functional protein, due to its recruitment into the aggregates, results in the failure of crucial cellular processes[78-80].

Recent experimental evidence suggests the formation of neurotoxic oligomers as a key pathological event not only in PD but also in other synucleinopathies[81, 82]. Although the molecular basis for oligomer neurotoxicity is still elusive, oligomeric species likely have a negative impact on the UPS machinery and on mitochondrial function[83]. These species also seem to trigger cellular defence pathways, such as the stress-response and caspase activation[84]. The aggregation of aSyn and the putative gain of toxic function may contribute to haploinsufficiency by entrapping the wild type aSyn protein, thus reducing the amount of available functional aSyn [80].

Therefore, in neurodegenerative diseases, it appears that the symptoms arise from the destruction of cells by a "gain of toxic function" that results from the aggregation process itself or by a combination of the "gain of toxic function" and a "loss of normal function" of the proteins in the cell [78-80].

1.2.1.2. Alpha-synuclein phosphorylation

Several PTMs have been described as modulators of aSyn aggregation, including phosphorylation[85], ubiquitination[86], sumoylation[87], nitration[88], enzymatic cross-linking, and C-terminal truncation[60, 88]. Phosphorylation at the aSyn serine 129 (pS129) is the dominant pathological modification of aSyn, and has emerged as a defining hallmark of PD and related synucleinopathies [85, 89-91]. Although aSyn is phosphorylated at low levels under physiological conditions, a striking 90% of aSyn appears to be phosphorylated on S129 in LBs and inclusions isolated from brains of patients who died with PD, multiple system atrophy (MSA), dementia with Lewy bodies (DLB), or other synucleinopathies [85, 89-92].

In addition to S129, serine 87 (S87), tyrosine 39, 125, 133 and 135 (Y39, Y125, Y133 and Y135) are also phosphorylated [93]. pS87 is increased in brains of transgenic models of synucleinopathies and human brains from AD, DLB and MSA patients[94].

The phosphorylation sites in aSyn are highly conserved and, with the exception of S87 and Y39, are located in the C-terminal region, suggesting important roles for these modifications in regulating aSyn interaction with other proteins, ligands, metals and small molecules[60] (Fig. 2). Accordingly, increasing evidence from pathological, genetic, animal model, biochemical and biophysical studies suggests that phosphorylation of aSyn at one

or multiple sites may play an important role in regulating its structure, membrane binding, oligomerization, fibril formation, LB formation and neurotoxicity *in vivo* [85, 89]. However, the significance of pS129 in the pathogenesis of synucleinopathies is still unresolved.

In vitro studies demonstrated that pS129 and pS87 significantly inhibits aSyn fibrillization [94, 95 96]. In rodent and *Drosophila* models of PD, pS129 promotes aSyn oligomerization but does not influence inclusion formation [97, 98]. Accordingly, abolishing pS129, using the phospho-resistant aSyn form S129A, promotes aSyn fibrillization and inclusion formation, favouring the hypothesis that pS129 results in kinetic stabilization or accumulation of toxic aSyn oligomers, in fly and rodent models[97, 98].

Concerning aSyn toxicity, pS129 accelerated neuronal loss in *Drosophila*, suggesting a toxic effect for aSyn pS129 [97]. Contrarily, PLK2, a major contributor of aSyn S129 phosphorylation, was shown to be protective in yeast [99, 100], *Caenorhabditis elegans* and rat neurons [101]. Consistently, over-expression of phospho-resistant aSyn form S129A in the brain of rat led to increased aSyn toxicity [102, 103], supporting the hypothesis that aSyn pS129 is protective. Nevertheless, whether pS129 promotes or prevents aSyn aggregation and toxicity, and the pathways affected by pS129, remains largely controversial[96, 104, 105].

1.2.1.3. Oxidative stress and mitochondrial dysfunction

Redox reactions are the basis for numerous biochemical pathways and cellular chemistry, biosynthesis and regulation. They are also important for understanding biological oxidation and radical/antioxidant effects [106].

A crucial advance of scientific interest in the field of oxygen toxicity and free radicals occurred when Fridovich described in 1969 [107] the existence of superoxide dismutase (SOD) in almost all aerobic cells, leading to the description of the superoxide theory of oxygen toxicity, which became the focus of much research and debate associated with ageing, development, diseases and cell signalling [106].

Oxygen-derived pro-oxidants can be classified as radical and non-radical oxygen metabolites, commonly named reactive oxygen species (ROS), which can cause damage to biological targets such as lipids, DNA, proteins and on the defending systems of the cell These are composed of enzymes and reducing equivalents, defined as antioxidants[54].

The organism must continuously confront and control the presence of both oxidants and antioxidants. This balance, often referred to as the redox potential, is specific for each organelle and biological site, and any interference of the balance in any direction might be deleterious for the cell and organism.

An increased generation of ROS or reactive nitrogen species (RNS), collectively known as RNOS, can occur via several cellular insults, including ultraviolet irradiation, redox-cycling of quinones, the metabolism of xenobiotics, ageing, environmental mitochondrial toxins, and mutant toxic proteins: e.g. Aβ in AD, mutant huntingtin in HD, aSyn in PD, SOD1 loss of function mutant in ALS [106].

Oxidative stress plays a central role in the pathology of PD as it arises during dopamine metabolism and during oxidative phosphorylation [108, 109]. Moreover, reduced complex I activity, observed in PD, increases ROS production [110-112] (Fig. 4).

Metabolism of dopamine leads to the formation of several cytotoxic molecules, including superoxide anions, dopamine–quinone species, and hydroxyl radicals [113, 114]. Dopamine breakdown may occur spontaneously in the presence of iron, or can be catalysed by monoamine oxidase (MAO) in a reaction that generates hydrogen peroxide [20].
Normally, cells scavenge these deleterious molecules using several antioxidant systems. For instance, reduced glutathione (GSH) peroxidase, oxidized glutathione, GSH-S-transferase, SOD and catalase[20]. In PD, however, an abnormal increase in the production of ROS might tilt the balance between production and elimination, leading to enhanced oxidative stress [20] which may also contribute to aSyn pathology in PD. In fact, oxidative modified aSyn is more prone to aggregation than the native protein[115]. Additionally, aSyn protofibrils may cause toxicity directly, inducing an oxidative stress that subsequently disables UPS by several mechanisms: reduction of ATP levels, inhibition of the proteasome, and oxidative modification of Parkin, which results in an increasing accumulation of aggregates, creating a vicious cycle of ROS and aggregates production. Furthermore, elevated aSyn expression can itself cause oxidative stress[116]. There is accumulating evidence for impaired oxidative phosphorylation and decreased complex I activity in PD, which leads to ROS formation and oxidative stress[117]. In parallel, aSyn expression induces the production of ROS, loss of the mitochondrial membrane potential, leading to opening of

the mitochondrial permeability transition pore (mPTP), release of cytochrome C from the intermembrane space to the cytosol, and activation of mitochondrial-dependent apoptosis, resulting in caspase activation and cell death[118], in yeast cells [119, 120] and human fetal dopaminergic neurons [121]. Suggesting that aSyn triggers the apoptotic cell death program.

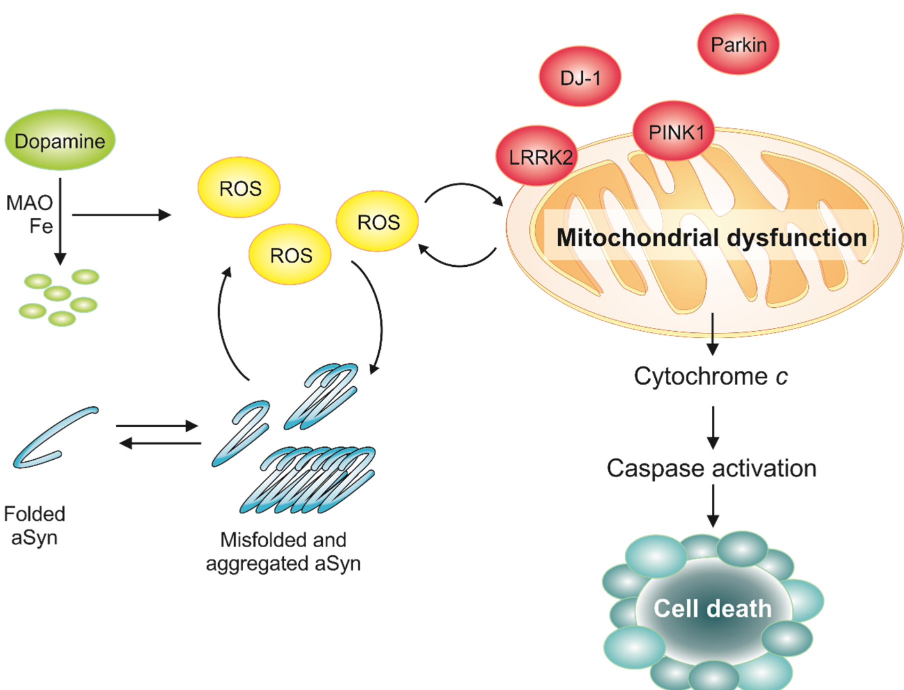

Figure 4: The interplay between aSyn aggregation, oxidative stress and mitochondrial dysfunction. Oxidative stress plays a central role in PD and it is a recognized trigger of aSyn misfolding and aggregation. In parallel, aSyn overexpression and protofibrils induces the production of ROS, creating a vicious cycle of ROS and aggregates. Reactive oxygen species (ROS) can be generated in the brain during the metabolism of dopamine. Dopamine breakdown may occur spontaneously in the presence of iron (Fe) or can be catalysed by monoamine oxidase (MAO), leading to the formation of several cytotoxic molecules and aSyn aggregation. Mitochondria have also been implicated in PD, impaired oxidative phosphorylation and decreased complex I activity was observed in PD, which leads to ROS formation and oxidative stress. Moreover, normal mitochondrial function is compromised by loss of function of LRRK2, PINK1, Parkin and DJ-1, leading to ROS production and aSyn aggregation. These genes are associated with early-onset Parkinsonism. The generation of ROS and concomitant cytotoxicity, leads to loss of the mitochondrial membrane potential, resulting in release of cytochrome *c* from the intermembrane space to the cytosol, and activation of mitochondrial-dependent apoptosis, ultimately resulting in caspase activation and cell death.

Normal mitochondrial function is notably compromised by loss of function of LRRK2, PINK1, Parkin and DJ-1, resulting in early-onset Parkinsonism[118]. Concomitantly, there is evidence that these proteins might have neuroprotective effects against mitochondrial dysfunction, although their exact site of action remains unknown [55, 118] (Fig. 4).

PINK1 is a serine/threonine kinase located in mitochondria, it appears to phosphorylate specific mitochondrial proteins exerting a protective cellular function. It has been shown to inhibit the release of cytochrome C following ceramide-induced stress [56]. Although the exact pathophysiological role of PINK1 is not clear, disruption of this kinase in *Drosophila* increased the vulnerability to oxidative stress and mitochondrial morphological defects, ultimately resulting in a gradual loss of dopaminergic neurons [46].

Parkin is an E3 ubiquitin-ligase, besides labelling specific substrates for degradation, it can control sorting and trafficking of proteins. Furthermore, observations made in cellular and transgenic models suggest a role for Parkin in mitochondria [46, 122], a hypothesis strengthened by its physical and genetic interaction with PINK1 [46, 122].

It was shown that Parkin acts as an effector of PINK1 affecting mitochondrial function [46, 123], since the phenotypes induced by loss of PINK1 were rescued by overexpression of Parkin, but the inverse was not observed [46, 123]. *In vitro* studies further demonstrated Parkin interaction with LRKK2 [124]. LRRK2 is a kinase that localizes at membranous and vesicular structures, including mitochondria [125]. Recent studies reported its function in the regulation of membrane dynamics relevant to endocytosis, mitochondrial morphology and function [43, 44]. Continuing to explore the role of PD associated genes with mitochondria, studies reported a physical interaction of Parkin and PINK1 with DJ-1[126, 127]. DJ-1 was shown to confer protection against ROS[128] and very diverse functions has been assigned. Although, the presumed anti-oxidative properties of DJ-1 are probably the most relevant for PD, chaperone activity and autophagy regulation have also been ascribed to this protein [41, 126].

The discovery of new therapies, preferably natural blood-brain barrier (BBB) permeable products with protective capacity, including food supplements which could help preventing/delaying the build-up of toxic aggregates, is then an important issue to be

addressed. The beneficial effects of certain (poly)phenols (e.g. catechins and resveratrol) on PD and aSyn oligomerization are well documented [129-134].

1.2.1.4. *Quality control systems*

Aggregation of misfolded proteins appears to be a complex physical-chemical process, being highly regulated by the cellular environment and modulated by various cellular components, as molecular chaperones, proteolytic enzymes and other factors[135]. Failure of such regulatory mechanisms is likely to be a major factor in the onset and development of misfolding diseases. Thus, proteostasis is a central event in the pathobiology of several disorders[136]. When the accumulation of unfolded proteins surpasses the capacity of the endoplasmatic-reticulum (ER) to cope with the protein load, diverse quality control mechanisms actively sequester and degrade these proteins [137-139]. The quality control mechanisms are conserved from yeast to mammalian cells [140] and the unfolded protein response, triggered upon ER stress, is one of the most important regulators. It leads to the transcription of genes related with restoration of protein-folding homeostasis, including chaperones, protein degradation and secretion pathways [137, 140].

Strong evidence on the involvement of the quality control systems in neurodegeneration came from genetic studies of familial PD cases[141]. PD causative mutations were identified in the UPS related genes, Parkin [142] and UCH-L1 [143], encoding an ubiquitin E3 ligase and an ubiquitin hydrolase, respectively. Homozygous mutations in the DJ-1 gene product, a redox-sensitive molecular chaperone[144] involved in normal UPS function[126], have also been described in families with PD inheritance[145].

The involvement of molecular chaperones on aSyn toxicity came from the observation that enhanced aSyn inclusion formation was observed upon deletion of individual chaperones in yeast [146]. Concomitantly, a mild heat shock, treatment with geldanamycin (a heat shock response activator), or overexpression of the chaperones Hsp70 [119], Jem1 or Hsp90 [147], protected yeast cells against aSyn-induced ROS and subsequent toxicity. The involvement of chaperones in PD was also supported by evidences obtained with neuronal cell lines [148], and transgenic *C. elegans* [149], flies [150] mice [151].

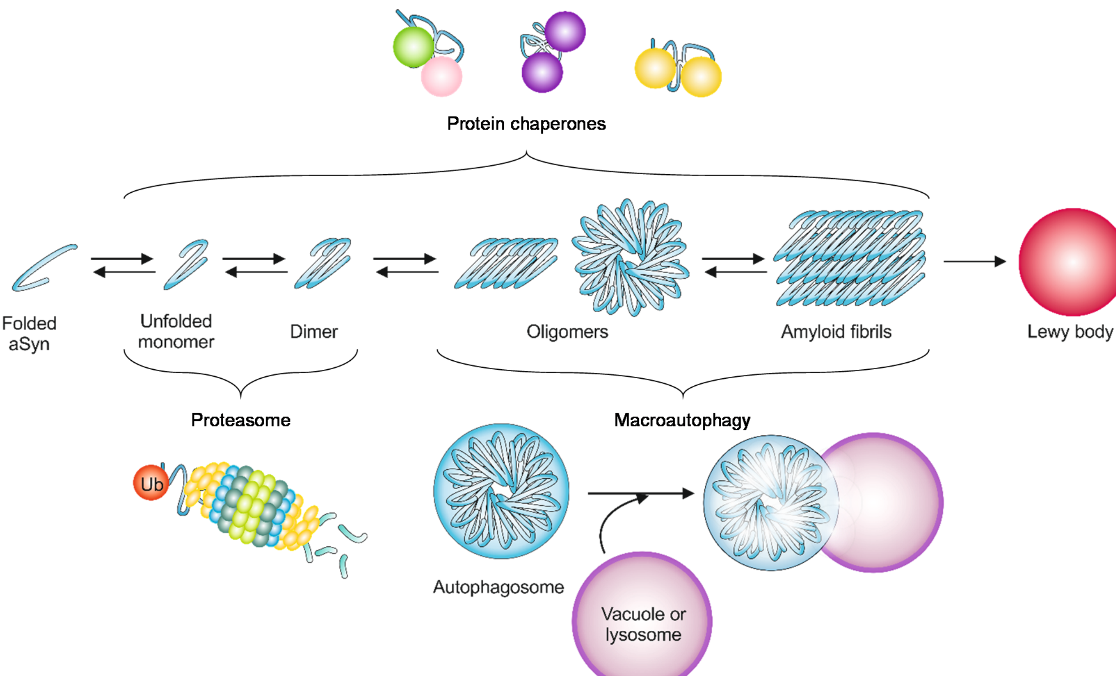

Figure 5: Cellular quality control systems are involved in cytoprotection at different stages of aSyn aggregation. aSyn can misfold and form pathological oligomers that fibrillize and deposit into larger aggregates, ultimately forming LBs. In healthy cells, the cellular quality control systems are able to counteract this cascade of events. The first steps of aggregation can be prevented or reversed by promoting the degradation of misfolded proteins by the ubiquitin-proteasome system while the later ones are counteracted by degradation mediated by macroautophagy. Chaperones can aid the correct conformation of proteins in the several states of aggregation.

Finally, the heat shock-induced protective mechanism may involve Hsp104 and its co-chaperones, which were described to relieve cells from ER-stress [152]. It was shown that Hsp104 degraded aSyn in a concentration-dependent manner and decreased aSyn fibrillation *in vitro* [153-155]. In agreement, Hsp104 antagonized aSyn aggregation and reduced dopaminergic degeneration in a rat model of PD [156]. It was shown that at endogenous levels the presence of Hsp104 had a deleterious effect on aSyn aggregation, and deletion of Hsp104 in yeast expressing WT or mutant aSyn resulted in lower oxidative stress, cytotoxicity, increased cell viability and rescue of endocytotic defects [157]. Nevertheless, another study reported that it is possible to reprogram Hsp104 to rescue aSyn proteotoxicity in yeast by mutating single residues [158]. These potentiated Hsp104 variants enhanced

aggregate dissolution, restored protein localization, suppressed proteotoxicity [158], and in a *C. elegans* PD model attenuate dopaminergic neurodegeneration[158].

aSyn expression promotes the impairment of the proteasome, as described in the pioneer study of the yeast PD model[159]. The reduced proteasome activity was found to be the result of a deficient proteasome composition[160]. Furthermore, the failure of the UPS enhanced aSyn toxicity[161, 162] and led to the accumulation of inclusions[163], either by impairing the UPS using proteasomal mutants [161, 162] or the chemical inhibitor, lactacystin [163]. Despite the clear evidence of proteasome dysfunction in PD, a recent study reported that the degradation of aSyn aggregates is more dependent on autophagy than on proteasome function, in the yeast PD model[164], suggesting that the proteasome is more likely to be responsible for the degradation of soluble forms of aSyn, and that there is a complex cross-talk between the different proteolytic pathways involved in aSyn degradation[165] (Fig. 5).

Autophagy involves the formation of an autophagosomal vesicle that transports the misfolded and aggregated proteins to degradation, in the lysosome in higher eukaryotes or the vacuole in yeast. The term autophagy was coined approximately 50 years ago and the molecular insights were significantly explored after the discovery of autophagy in yeast subjected to starvation [166 167]. Autophagy is regulated by the kinase "target of rapamycin" (TOR) [168]. Lst8, a TOR interacting protein was identified as an aSyn toxicity modulator in yeast[169]. Moreover Ypk9 (the yeast orthologue of ATP13A2), a vacuolar P-type ATPase, was identified as a suppressor of aSyn toxicity [169] and aggregation[101]. PD-associated mutations in ATP13A2 appear to cause a loss-of-function as the protein no longer localizes at the lysosome [170]. The role of this ATPase in neuropathology is not completely understood, but its putative lysosomal function may have important consequences for autophagy-mediated clearance of protein aggregates and organelles. Concomitant with the beneficial influence of autophagy in aSyn toxicity, rapamycin treatment, which induces autophagy by inhibiting TOR, was reported to reduce aSyn inclusions [163]. These observations are in line with experiments indicating that under pathological conditions, autophagy is required for the degradation of aSyn aggregates, rather than the proteasome[164]. However, the autophagic flux is disrupted in PD patients cells, due to a deficient mitochondria-dependent traffic[171].

There are controversial findings regarding the role of autophagy on aSyn toxicity. A study reported that rapamycin treatment lead to increased aSyn toxicity in yeast[169]. It was also shown that WT or A53T mutant aSyn were not able to enter the vacuole and promoted vacuolar fusion defects in yeast[146]. Additionally, aSyn-mediated mitophagy, specific degradation of mitochondria through autophagy, was reported to be deleterious in aged yeast cells [172]. To intensify the discussion, aSyn LB-like aggregates resisted degradation and impaired autophagy in cellular models [172].

It is clear that the interplay between autophagy and aSyn toxicity and aggregation is still elusive and needs to be explored.

1.3. Yeast as a model of PD

Saccharomyces cerevisiae, also known as baker's yeast, is one of the most versatile biological systems model. This unicellular eukaryotic organism is extremely useful for molecular biologists and it has been extensively used in the study of numerous complex and devastating disorders, such as HD and PD [173].

Multiple characteristics make this simple eukaryote a model system of choice. Despite the obvious absence of a nervous system, basic mechanisms and pathways underlying neurodegenerative diseases, such as mitochondrial dysfunction, transcriptional deregulation, trafficking defects and proteasomal dysfunction, are highly conserved between yeast and humans, enabling detailed studies of the molecular events involved in those conditions. In fact, several remarkable insights into the understanding of brain diseases have been recently achieved with yeast models[84, 173, 174]. To develop disease models, it is essential that some relevant aspects of the disease phenotype are reiterated. It is possible to study directly a gene function implicated in a disease, if provided a yeast homolog. On the other hand, if the gene is absent in yeast, and causes disease by a toxic gain-of-function in humans (eg. *SNCA*), it can still be modelled *via* the heterologous expression of the human[84].

Notably, several PD associated genes, such as aSyn, LRRK2, Parkin, DJ-1, VSP35, EIF4G1 and ATP13A3, haven been successfully expressed in yeast, by heterologous or homologous expression, and represent the yeast PD models currently available (Fig. 6).

Nevertheless, the yeast PD model most used is based on the heterologous expression of aSyn and within this models various phenotypes can be achieved, depending on the expression system, given that this affects the level of aSyn expression [159, 175, 176]. This feature has been also explored according to the objectives of the studies.

The use of multicopy plasmids revealed that yeast cells reduce the average plasmid copy number in order to reduce aSyn expression and toxicity [159]. To avoid this, insertions of the aSyn coding sequence in the yeast genome enabled more stable expression and the levels of toxicity could be manipulated by varying the number of copies of the aSyn cDNA inserted in the genome [176, 177]. The use of a galactose-inducible promoter provided additional control for the synchronous induction of expression of aSyn, avoiding the negative pressure during routine cell manipulations.

Using these various expression systems, several genetic modifiers (enhancers and suppressors) of aSyn toxicity were identified in genetic screenings in yeast [175, 176].

In other studies, yeast cells expressing different levels of aSyn, hence displaying different levels of cytotoxicity, revealed the involvement of multiple cellular pathways in the toxicity [159, 176-181].

The drug discovery process for synucleinopathies has been delayed by the elusive nature of the mechanisms leading to neurodegeneration and lack of reliable model organisms to screen for compounds. Moreover, aSyn is a ”natively unstructured protein”, missing a defined secondary structure under physiological conditions, which constitutes an additional challenge for drug discovery[73]. As a major effort to overcome these difficulties several phenotypic high-throughput assays have been developed. These are cell-based assays that recapitulate phenotypic and functional aspects of disease, focused on viability/toxicity readouts, leading to the identification of small molecules and also potential novel drug-targets. However, it is essential to scrutinize the mechanism of action of the small molecules and candidate compounds. Here yeast cells offer a remarkable advantage, it allows target and mechanisms identification thought diverse and complementary genetic approaches, empowering the development of pre-clinical candidates. Furthermore, several cell-based assays targeting key molecular aspects of PD, such as aSyn cytotoxicity, aggregation, proteasome impairment and ER-to-Golgi trafficking have been developed in

yeast[159, 176, 182]. All of these processes are well conserved between yeast and higher eukaryotes, including mammals.

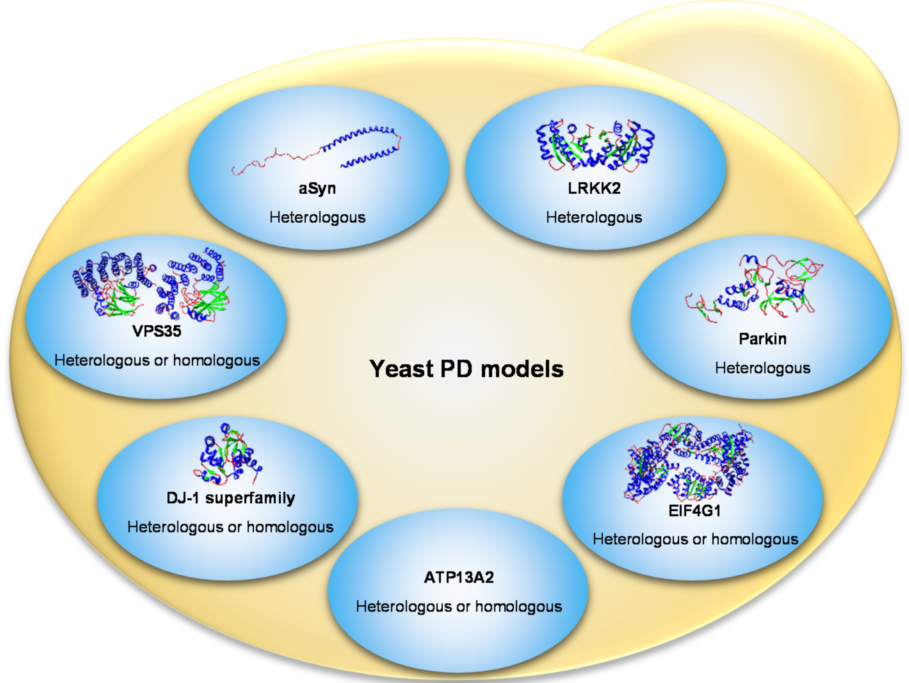

Figure 6: Yeast models of Parkinson's disease. Proteins associated with PD are shown, as well as the type of expression (heterologous or homologous). The structure of the proteins are represented for aSyn, LRRK2, Parkin, EIF4G1, DJ-1, and VPS35 (Protein Data Bank ID: 1XQ8, 2ZEJ, 4I1H, 2VSX, 4OQ4 and 2R17 respectively. ATP13A2 structure is not determined).

The main drawback for phenotypic high-throughput assays using stable immortalized cell lines is the resistance to proteotoxic insults, resulting in relatively low aSyn toxicity. This resistance is in part caused by the reduced apoptotic mechanisms necessary to enable proliferation in culture. However, assays that monitor cell viability through mitochondrial potential or membrane leakage could be of use in low toxicity models, however extensive optimization is required. Primary rat neurons infected with aSyn-expressing lentivirus, are more stable and sensitive but they also present technical limitations related with virus and cell quantities, besides being costly and labour demanding. Indeed, the lack of published cell culture toxicity screens reflects the difficulty in their employment in high-throughput formats[183, 184].

Yeast has numerous advantages at the early stages of the drug development process, in comparison to mammalian cells and animal models. Notwithstanding the obvious lack of a nervous system the simplicity of yeast, missing the complex neuronal communication with other cell types makes it ideal to study early-onset events in disease pathology. Some useful properties of yeast include the ease of experimental and genetic manipulation, low cost, functional similarity to higher eukaryotes and fast growth. Additionally, several major drugs have hit the same targets and elicit the same responses in yeast as they do in humans, including statins, methotrexate, omeprazole, tacrolimus (FK506) and bortezomib (Velcade) [183, 185]. The yeast platform enables rapid screen and analysis of therapeutic targets, providing a direct and important linkage between pathways and chemicals. Several examples of pharmacological discoveries using yeast-based assays have been reported. These have been performed in 384- and 1536-plate format, illustrating their high-throughput potential [186].

Nevertheless, it is important to acknowledge that yeast has its limitations, as with all model organisms. It is a unicellular organism, some genes important in neurodegeneration may be absent and it has a cell wall, which might constitute a barrier to some molecules. Yet, this last undesirable attribute can be attenuated by genetic manipulation of the efflux pump system or the ergosterol biosynthesis, reducing the capability of yeast cells to export drugs or by increasing yeast cells permeability, respectively. Though, the unparalleled discovery characteristics of yeast outweigh any potential drawbacks, particularly since many genes tied to neurodegenerative disorders are ubiquitously expressed and highly conserved [187].

Yeast is considered a robust primary drug-screening platform to filter for compounds with cytoprotective activity, for further complementation with assays in more physiologically relevant models. Approaches involving the sequential use of different model systems, starting with simpler cellular models and ending with more complex animal models, already resulted in the discovery of promising small molecules with therapeutic potential (described below). Recently, it was established a yeast-to-human discovery platform for synucleinopathies, where genes and small molecules identified in yeast were validated in

PD patient derived neurons. Subsequently, yeast was used for clarification of the mechanism of action, due to yeast unmatched genetic tools [188].

Ultimately, yeast-to-human discovery and validation platforms will provide more significant findings into disease pathology. By iteratively moving between simple cellular models and patient derived cells we will be able to elucidate mechanisms and evaluate patient-specific drug targets, this will allow to pursue more significant animal and clinical trials in order to overcome neurodegenerative diseases.

1.3.1. Testing small molecules and natural compound in yeast

The reduced number of reported high-throughput drug screens highlights the difficulty in establishing robust, meaningful PD models amenable to screening. Yeast, despite its obvious differences from neuronal cells, has actually provided potentially beneficial lead compounds against aSyn toxicity.

The yeast discovery platform has been used as an approach to understand basic mechanisms of protein aggregation and toxicity. The events leading to protein oligomerization are likely amenable to modulation by small molecules. Thus, notably, yeast has been used to screen for small molecules that can inhibit aSyn aggregation and toxicity.

Screening of large libraries of compounds lead to the identification of aSyn toxicity suppressors in yeast. In a large-scale genetic screen, ~115 000 compounds were tested for the ability to rescue aSyn toxicity at micromolar concentration and a class of structurally related 1,2,3,4-tetrahydroquinolinones were identified[177]. These compounds reduced the formation of aSyn inclusions, re-established ER-to-Golgi trafficking, and ameliorated mitochondria defects induced by aSyn. The targets were further confirmed in nematode neurons and in primary rat neuronal midbrain cultures. Interestingly, these compounds also rescued rotenone toxicity in neuronal cultures, a toxin used to study mitochondrial deficit in PD[177].

The ease of manipulation makes yeast a suitable tool to explore unconventional compounds and its mechanisms. Mannosylglycerate, a compatible solute typical of marine microorganisms thriving in hot environments, was found to reduce aSyn aggregation in a yeast model of PD[189]. Latrepirdine, a phase II clinical trials drug, was identified as protector

against aSyn by inducing its degradation through autophagy, representing a novel scaffold for discovery of robust pro-autophagic/anti-neurodegeneration compounds[190].

A novel class of molecules, cyclic peptides (CPs), was also screened in yeast[191]. CPs are natural-product-like chemicals with potent bioactivity. Yeast was exploited to express a plasmid-derived self-splicing intein that liberates a CP. This approach enabled the scale-up of high-throughput screens to 10–100 times the size of a typical small molecule screen. A pool of 5 million yeast transformants were screened and two related CP constructs with the ability to reduce aSyn toxicity were identified. These cyclic peptide constructs also prevented dopaminergic neuron loss in a nematode model of PD[191].

Small molecules have been the centre of drug high-throughput screenings, and amongst them phytochemicals have emerged as attractive molecules in the context of neurodegeneration. It is largely accepted that dietary products such as green tea, small fruits and even olive oil have health benefits, however the major advances regarding its mechanisms and targets were only achieved in the last decade. The yeast allied with animal and chemical studies has significantly contributed for these discoveries.

The first small compound high-throughput screen in yeast tested ~10 000 compounds and identified a group of protective flavonoids, quercetin and epigallocatechin gallate (EGCG), which protected aSyn-expressing yeast in the presence of iron[192]. The protection promoted by these compounds was further analysed. The positive effect was virtue of its anti-oxidant and metal-chelating activities. Importantly, (poly)phenols, and particularly quercetin and EGCG, have been proven beneficial in cellular and animal models of PD [193, 194].

The advances in biochemical tools and the multidisciplinary teams also gave a major contribution to drug discovery. In fact, the green tea benefits have been deciphered by combining HPLC fractionation in a microplate format with yeast screening and parallel electrospray mass spectrometry[195]. This integrated process enabled the rapid assess of the efficacy of fractions and systematically target their bioactive constituents. The green tea metabolites were individually examined for their pharmacological effects and interestingly, the protective properties of *Camellia sinensis* lied on the combination of multiple catechin

metabolites[196]. This study emphasizes the prominence of yeast high-throughput screenings to dissect natural extracts and explore the numerous synergistic effect of its metabolites.

Clues for candidate compounds for aSyn pathology can also be obtained from screenings in other neurodegenerative disease models, as the case of ALS yeast model, based on the expression of the protein TDP-43 [197]. The yeast powerful genetics was used to identify multiple protective 8-hydroxyquinolines, which are natural plant alkaloids [197]. Some of these compounds were also protective in aSyn yeast and nematode models. Its protective mechanisms were related with its ionophores and intracellular metal chelation activity [197]. From this screening N-aryl benzimidazole (NAβ) proved more potent and effective against aSyn toxicity than TDP-43. Thus, the yeast aSyn platform was explored to identify NAβ mechanisms. It reversed diverse phenotypes induced by aSyn, including the accumulation of aSyn inclusions, the generation of ROS, the block of ER–Golgi trafficking and the nitration of proteins [198]. Moreover, this compound was used in an iterative yeast-to-human neuron platform to understand how translational its targets were [181].

Taken together, identifying disease therapies is an incredible challenge. Nevertheless, rapidly improving methodologies and iterative processes, allied with an evolving mechanistic understanding of disease, is nurturing more interdisciplinary approaches to research and fostering drug discovery, with the ultimate goal of discovering novel therapeutics for humans.

2. (Poly)phenols

Phenolic compounds, commonly referred to as polyphenols constitute one of the most extensive and ubiquitous group of secondary metabolites in the plant kingdom. It is estimated that more than 8000 compounds have been isolated and described [199]. These compounds are characterized structurally by the presence of, at least, one hydroxyl functional group (-HO) linked to an aromatic ring. Polyphenols are usually conjugated to sugars, carboxylic and organic acids, amines, lipids and other phenols. They are classified according to the number of phenols rings and the structural elements that bind these rings[199]. The main polyphenol classes are represented in Fig. 7.

Some compounds that do not present characteristic structure of polyphenols are commonly integrated in the group of polyphenols as "honorary", such as phenolic acids or stilbenes. For this reason, recently the term polyphenols has been written as (poly)phenols, as shown from hereafter[200-202].

In plants, (poly)phenols, as secondary metabolites, do not participate in the primary metabolism processes, e.g. photosynthesis or respiration. However, they have diverse functions: coloration of leaves, flowers and fruits; defence from pathogens and herbivorous predators; anti-microbial and anti-fungal activities; screening from solar UV radiation; chelation of toxic heavy metals; protection from free radicals generated during photosynthesis[203].

(Poly)phenols have attracted much attention in the last decades due to their health-promoting properties. Epidemiological and clinical studies found the benefits of (poly)phenols on heart disease, cancer, atherosclerosis, obesity, allergies, gastrointestinal, neurological and liver diseases [204, 205].

2.1. (Poly)phenols in neurodegenerative diseases

Over the last 35 years natural products have been use as drug leads. Between the years 2000 to 2010 50% of all small molecules were produced from natural products [206] and from 2005 to 2007 13 new natural products related drugs were approved by FDA or equivalent agencies [207]. Such as galantamine, from the plant genera *Galanthus* [208], and huperzine, from moss *Huperzia serrata* [209]. They are potent inhibitors of acetilcolinesterase (AChE) and are considered for clinical use in the treatment of AD. Among the plant species with promising effects in dementia patients are *Salvia officinalis* L., *Salvia lanandufolia* Vahl., *Melissa officinalis* L., *Crocus sativus* L., and the extensively studied *Ginkgo biloba* [210]. Over 100 natural products derived compounds are currently under clinical trials and most are derived from plant leads [207]. As expected natural products have inspired many developments in organic chemistry, originating analogues with improved pharmacological properties and several libraries based on alkaloids, polyketides, terpenoids and flavonoids [211, 212]. Thus, (poly)phenols and other natural products have emerged as promising candidate compounds

in the treatment of neurodegenerative diseases, using pharmacological or nutritional approaches.

Ever since the James Joseph's group observed that a diet supplemented with (poly)phenols from strawberry and spinach prevented deficits in motor and cognitive behaviour in aged rats[213, 214], the neuroprotective potential of (poly)phenols have been explored. The common features of neurodegenerative pathogenesis include cytokine changes, genetic alterations, immunomodulation, inflammation, mitochondrial dysfunction, oxidative stress, prions, and protein dysfunction[78]. Research has shown that (poly)phenols target the pathological manifestations of neurological disorders with their ability to cross BBB, as they control neuronal disease pathogenesis at a molecular and symptomatic level by targeting these common features of neurodegeneration[213, 214 132, 206 215 216].

Although (poly)phenols are not considered essential nutrients they are wide spread in food, and the total (poly)phenols dietary intake could be as high as 100-150 mg per day, which is much higher than that of all other classes of phytochemicals and known dietary antioxidants [217]. For perspective, this is one order of magnitude higher than the intake of vitamin C and two orders of magnitude higher that the intakes of vitamin E and carotenoids [218, 219]. Their main dietary sources are fruits and plant-derived beverages such as fruit juices, tea, coffee, and red wine. Vegetables, cereals, chocolate, and dry legumes also contribute to the total (poly)phenol intake [217].

In a nutritional approach, regular consumption of flavonoid rich-foods has been associated with better cognitive performance and decreased risk of cognitive decline in elderly population [220, 221]. The adherence to the Mediterranean diet, rich in (poly)phenols, is also associated with lower risk of developing AD, mild cognitive impairment [222], lower mortality in AD patients, delayed AD [223] and PD [224] onset. Intake of flavonoids was negatively correlated with dementia incidence, premature death due to dementia and disability costs, in a large-scale population study [221]. Furthermore, evidence supports a role of (poly)phenol-rich diets in cognitive, memory, learning vascular function improvement, in elderly people[133, 205]. Collectively, these reports favour the hypothesis that (poly)phenol-rich foods or supplements have a positive impact in neurodegenerative diseases.

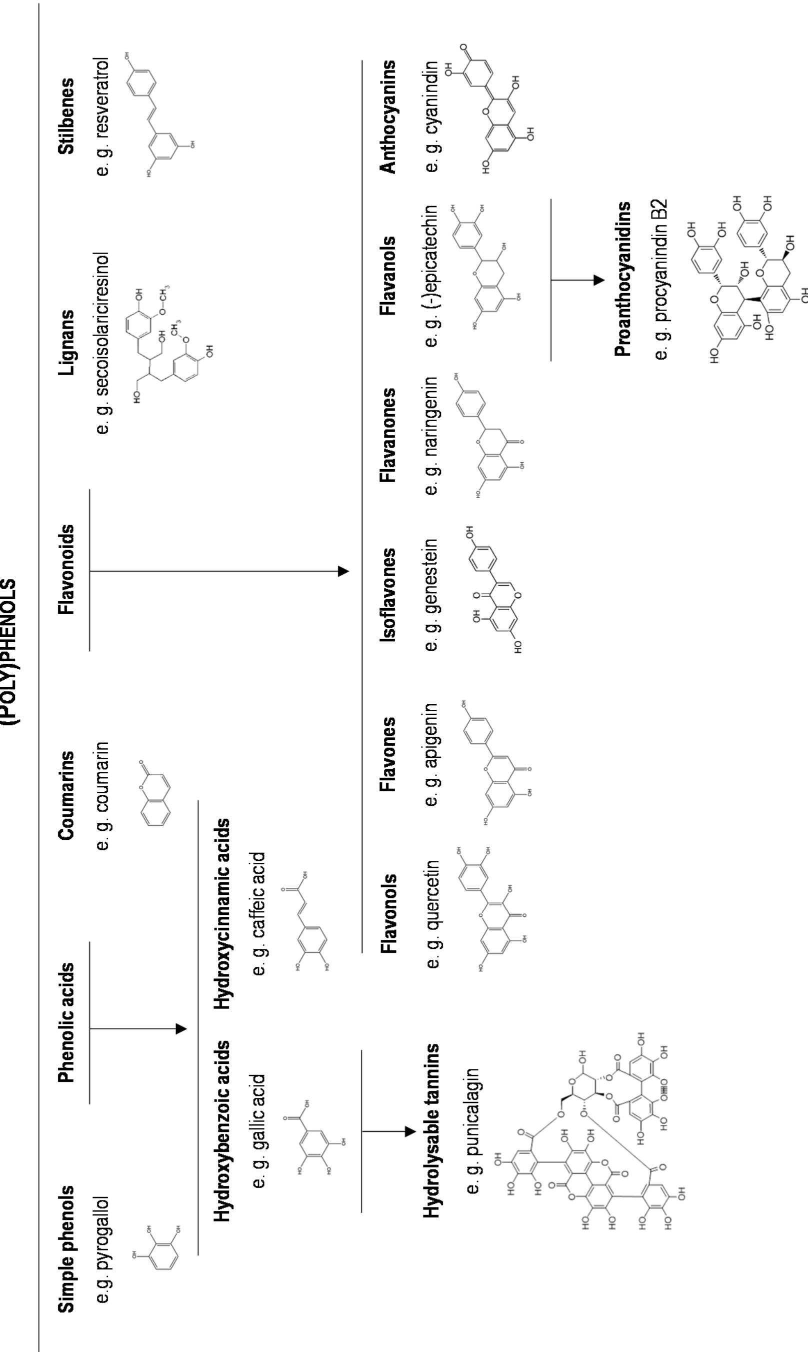

Figure 7: Classification and chemical structures of the main classes of (poly)phenols.

2.2. Neuroprotective mechanisms of (poly)phenols

Pure (poly)phenols or (poly)phenol-enriched extracts have been described as displaying neuroprotective activity. Those evidences arose from *in vitro*, cellular, animal, epidemiological and clinical studies[133, 225]. Although, the clinical studies are more reliable and the closer approach to human physiology, they are time and cost consuming. The *in vivo* and cellular assays constitute an unparalleled tool to dissect the mechanisms of action and for genetic and compound screening assays. On the other hand, the *in vitro* assays are crucial to understand the interaction of (poly)phenols with proteins, metals or other small molecules, by excluding the cell components interference. Some of these findings are summarized in this section. However, most of the studies use pure compounds, missing the synergies between different (poly)phenols and the physicochemical alteration they suffer upon digestion and further metabolism.

For many years, (poly)phenols were thought to protect cell constituents against oxidative damage through scavenging of free radicals. However, this concept now appears to be an oversimplified view of their mode of action. There is an emerging acceptance that (poly)phenols, as well as their metabolites, exert modulatory actions in cells through direct interaction with receptors or enzymes involved in signal transduction, such as protein kinase and lipid kinase signalling pathways [226]. Moreover, several neurochemical mechanisms underlying the protective action of plant (poly)phenols have been described: iron chelating properties [192]; modulation of signalling pathways related with neuronal survival and differentiation [226, 227]; inhibition of neuropathological processes [228, 229]; and regulation of mitochondrial function [204, 230, 231].

One of the best studied source of plant (poly)phenols is *Ginkgo biloba.* Its extract (EGb 761) has been described to scavenged RNOS *in vitro* [232], protected from Aβ [233] and amyloid precursor protein (APP) [234] neurotoxicity in cell models. Moreover, it reduced neuro-inflammation by activating the silent mating type information regulation 2 homolog 1 (SIRT1) and supressing the nuclear factor kappa B (NF-κB)[233]. In AD transgenic mice, oral intake of *G. biloba* extract improved spatial learning and memory[235], decreased APP in the cortex [236] and inducible nitric oxide synthase (iNOS) expression[237]. In human trials most of the results were inconclusive[238], and long-term oral intake of *G. biloba* did not affect the prevalence of

cerebral Aβ deposition[239]. Nevertheless, recently positive results were obtained with *G. biloba* extract in ischemic stroke [240] and in children with attention deficit hyperactivity disorder[241].

Another well explored source of (poly)phenols is tea. Green and white tea digested metabolites inhibited AChE *in vitro*[242], with potential for AD therapeutics. Green tea scavenged RNOS, induced endogenous antioxidant defences preventing DNA damage [243], in cellular models. In animal models, green tea oral administration prevented protein/lipid oxidation[244], improved spatial learning[244], modulated glutathione levels and antioxidant enzyme activities[245], increased CREB activation[245], elevated BDNF and Bcl-2 levels[245] and improved cognitive and behavioural capacities [246] during aging and neurodegeneration. Moreover, green tea extract injected into the hippocampus of rat reduced aluminium chloride toxicity, associated with AD[247]. It also protected primary rat cortical neurons against Aβ-induced cytotoxicity[248]. A recent study provided the first evidence that tea (poly)phenols can alleviate motor impairments, dopaminergic neuronal injury, and aSyn aggregation in nonhuman primates[249]. The main studied (poly)phenols in green tea are catechins and epigallocatechin gallate (EGCG). Recently, green tea extract protected against deltamethrin-induced neurotoxicity in rat, after oral ingestion, through improving the oxidative status and DNA fragmentation[250].

Catechins increased neuronal viability by modulation of signal transduction pathways, cell survival genes and mitochondria functions, reduction of neurotoxins and Aβ toxicity in cell lines and primary cultures [246]. *In vivo,* oral administration of catechin prevented spatial learning and memory decline in aged mice [251], and increased the life span in a senescence-accelerated mice model [252]. It also ameliorated redox imbalance and limited inflammation in rats with cerebral ischemia, by oral administration[253].

EGCG prevented Aβ fibril formation *in vitro* [129] and inhibited caspase activation mediated by Aβ in hippocampal neurons, leading to increased viability[254]. In animal models, EGCG improved age–related cognitive decline and protected against ischemia/reperfusion (intraperitoneal administration)[255], brain inflammation (gavage administration) [256] and amyloidosis in AD mice models (intraperitoneal administration)[228]. Moreover, it rescued memory impairment induced by Aβ, through inhibition of the NF-κB pathway and mitigation

of oxidative stress in the brain, by oral administration[257]. Furthermore, EGCG oral intake restored mitochondria function in the brain hippocampus, cortex and striatum[258]. An EGCG diet rescued transgenic mice from morphogenesis defects, low brain-derived neurotrophic factor (BDNF) levels and mnemonic deficits[259]. It also exhibited neuroprotective effects by modulating neuroinflammation and attenuating neural damage in an *in vivo* model of MS (gavage administration)[260].

Epidemiology studies revealed that tea consumption was inversely correlated to incidence of dementia, AD and PD[261], prevalence of cognitive impairment in Chinese [262] and Japanese[263] elders, and incidence of PD in American[264], Finnish [265] and Singaporean[266]. In the human studies, green tea extract increased brain activity in the dorsolateral prefrontal cortex, an area involved in memory processing[267]. Recently, consumption of tea by increased the brain theta waves, suggesting a role in cognitive function, specifically alertness and attention in healthy volunteers[268]. Moreover, EGCG reduced cognitive deficits in a pilot study with Down syndrome individuals, with effects on memory recognition, working memory and quality of life[269].

Curcumin, present in curry spice turmeric, presented anti-inflammatory properties through inhibition of cyclooxygenase-2 (COX-2), suppression of iNOS and inhibition of NF-κB activation [270]. It also inhibited the formation of Aβ fibrils and destabilized preformed fibrils *in vitro* [271] and reduced Aβ plaques, oxidized proteins and Interleukin-1 beta (IL-1β) in AD transgenic mice, subjected to curcumin diet and carotid artery injection[271]. Notable, it was able to cross the BBB, target and disrupt existing plaques (tail vein injection)[215], and counteract HD aggregates formation when present in diet[272]. However, phase-I clinical trials demonstrated low bioavailability following oral intake[273], not excluding the possibility of a pharmacological approach to curcumin.

Rosemary phytochemical, rosemarinic, alleviated memory impairment mediated by Aβ, delayed disease onset and increases lifespan in AD [274] and ALS [275] mouse models after intraperitoneal injection. Carnosic acid, also present in rosemary, protected human neurons from oxidative stress, crossed de BBB and preserved GSH levels in mouse models of ischemia/reperfusion (intraperitoneal injection)[216].

Another source of (poly)phenols in the stardom is grape and its derivatives. Grape seed (poly)phenol extract reduced cognitive deterioration[229] and improved cognitive function [276], inhibited oligomerization of Aβ peptides[229], amyloid plaques and microglial activation in animals models of AD[277], after oral ingestion. Moreover, its incorporation in diet attenuated extracellular-signal-regulated kinases (ERK) 1 and 2 signalling in the brain, suppressing AD tau neuropathy[278]. Grape seed also exhibited potential in HD treatment, following oral administration [279]. Furthermore, grape (poly)phenols were able to cross the BBB, by labelling (poly)phenol with ^{14}C and administering them to rats by gavage[280].

A derivative of grape, red wine, increased spatial memory performance, cognitive deterioration and Aβ neuropathy in AD transgenic mice[281]. Moreover, in an AD mouse model, wine oral consumption reduced hippocampus neurodegeneration and prevented hearing impairment through modulating sirtuin activity [276, 282]. Resveratrol, present in grapes and red wine, exhibited great potential in neurodegenerative diseases prevention [276, 283, 284]. It reduced Aβ mediated accumulation of ROS and apoptosis in cell models[285]. In an animal model overexpressing APP, a resveratrol diet reduced the formation of amyloid plaques, without affecting APP levels[286]. In another AD mouse model injected with Aβ, resveratrol (intracerebroventricular injection) protected from Aβ neurotoxicity by inhibiting iNOS[287]. Resveratrol also exhibited a positive effect In PD animal models, it reduced neural inflammation by lowering mRNA levels of COX-2 and tumour necrosis factor (TNF-α) mRNA in the *substantia nigra* (oral administration) [288] along with attenuation of oxidative stress, lipid peroxidation, and protein carbonyl (intraperitoneal injection)[289]. Resveratrol protection was also shown to occur through modulation of NF-κB and SIRT1 pathways in cell models [285, 290]. In yeast, resveratrol mimics calorie restriction by stimulating Sir2 (the homologue of SIRT1), increasing DNA stability and extending life span by 70%[291]. In agreement, in animal models of MS, resveratrol oral intake was found to attenuate neural damage through SIRT1 activation [292], and prevented neural loss without immunosuppression [293]. In rat models of ischemic injury, intraperitoneal injection with resveratrol improved brain energy metabolism [294], along with the modulation of the release of neurotransmitters and neuromodulators[295]. Regarding HD, resveratrol also shown beneficial effect via SIRT1 activation in a transgenic mouse model, administered by oral gavage[296].

Berries have attracted much attention lately. They are known for its high content in (poly)phenols and have also shown beneficial effects in cellular and animal models of neurodegenerative diseases. Blueberry extracts increased Aβ clearance, inhibited its fibrillization and supressed microglia activation, in murine cell culture[297]. In neuronal cultures exposed to H_2O_2, blueberry juice, reduced cell death mediated by extracellular signal-regulated kinases and activated pro-survival pathways[298]. In animal models, a blueberry supplemented diet reversed age related deficits in spatial working memory through increase in cAMP response element-binding protein (CREB) activity, a transcription factor which binds to the promoter regions of many genes associated with memory and synaptic plasticity; increase in BDNF, involved in neuronal plasticity, survival and function; phosphorylation of hippocampal Akt, a survival pathway; activation of TOR and overexpression of Arc/Arg3.1[299], suggesting a role for (poly)phenols in the regulation of pathways involved in de novo protein synthesis. Additionally, blueberry anthocyanins were shown to cross the BBB, analysed in pigs fed with blueberries[300].

Intake of berries such as pomegranate, strawberry, blueberry and blackberry ameliorated several aspects of memory and learning [301], delayed age-related and cognitive behavioural deficits in rodent models [214, 302, 303]. Pomegranate juice oral intake also improved spatial learning, and reduced Aβ plaques in transgenic mice expressing APP[304].

Anthocyanins, a main (poly)phenolic class in berries, prevented neurodegeneration by modulation of several cellular pathways [305, 306]. An anthocyanin rich extract has been shown to affect NF-κB activity in microglia cells, accompanied by a down-regulation of iNOS and COX-2[307]. In cellular models expressing Aβ anthocyanins retained the cell viability, normalized mitochondrial potential and calcium level, decreasing neuronal cell death[308]. In rats receiving anthocyanins intragastrically, it reversed Aβ-induced effects on protein expression: mitochondrial apoptotic pathway (Bax, cytochrome C, caspase-9 and caspase-3) and AD markers (Aβ, APP, P-tau and BACE-1)[308].

Epidemiological studies showed that high anthocyanin consumption is associated with a reduction of the risk of developing PD[309]. It has been recently reported that anthocyanin metabolites are present in plasma in considerably high amounts[310].

Other (poly)phenols wildly distributed in fruits and vegetables, like quercetin (present in onions) have shown neuroprotective potential. Quercetin inhibited aSyn fibrillization *in vitro* [311], increased cell survival upon treatment with H_2O_2, hydroperoxide, tert-butyl hydroperoxide (TBHP), IL-1β and PD related toxins [312]. *In vivo*, quercetin oral intake improved memory and synaptic plasticity upon chronic lead exposure [313], protected against colchicine-induced cognitive impairment [314], improved motor function in a model of acute spinal cord injury (intraperitoneal administration)[315], reduced ischemic lesion (intraperitoneal administration)[193] and hippocampal neuronal death in a rat ischemia model (intraperitoneal administration)[316]. In PD *in vivo* models, quercetin oral administration showed to be neuroprotective by inducing antioxidant defences and ATPases[317]. Quercetin was also found to control immune response via modulation of IL-1β and TNF-α and reduced the proliferation of peripheral blood mononuclear cells isolated from MS patients[318]. Furthermore, quercetin has raised interest as therapeutic for cerebral ischemia due to its powerful inhibition of metalloproteinases (MMPs) and acid sensing ion channel 1a (ASIC1A) [319].

The diversity of (poly)phenol's targets makes them an attractive alternative for the treatment to multifactorial diseases, where several cellular pathways are disrupted. Although, there is a clear lack of clinical trials regarding (poly)phenols in the context of neurodegenerative diseases [133, 320], which must be met in the future.

3. *Plants used*

Plants are an immense reservoir of (poly)phenols and the diversity of Portuguese plants pharmacological/nutraceutical value is still poorly characterized. Thus, in this thesis the (poly)phenols from *Arbutus unedo* and *Corema album* were analysed for protective properties in Parkinson's disease cellular models (Figure 8).

3.1. Ericaceae family

Corema album L. and *Arbutus unedo* L. belong to the *Ericaceae* family, which comprises angiosperm plants, mostly calcifuge (lime-hating) that thrive in acidic soils. From the economical point of view, the plants from this family are used as ornamentals, due to the colourful inflorescences of their shrubs. Moreover, the fruits of some species are edible and

drupaceous, although only a few are produced commercially and marketed (e.g. blueberry, *Vaccinium myrtillus*) [321, 322].

3.1.1. Corema album

Populations of the *Corema* gender are a major vegetation component of fragile ecosystems, such as dry sand dunes in coastal regions and alpine areas, and thus are ecologically of great importance. *Corema* is distributed on both sides of the Atlantic Ocean, showing a typical amphi-Atlantic distribution pattern [323, 324].

C. album also known as Portuguese crowberry, occurs in the Iberia Peninsula, being autochthone in France. It has been cultivated as an ornamental plant, due to its fruits that look like little pearls [324]. They are edible, and in ancient times were known for its antipyretic and antiparasitic properties [324]. This species is still poorly characterized and the chemical composition of its berries was performed recently [323]. However, the studies regarding its bioactivities are scarce, *C. album* represents a reservoir of phytochemicals to be explored.

3.1.2. Arbutus unedo

A. unedo, commonly referred to as strawberry tree, is a perennial shrub, native to the regions of Mediterranean climate, which grows wild in the rocky coastline of southern Europe[325, 326]. Its fruits are edible, spherical with a diameter of about 2 cm, dark red, and consumed preferably in the autumn, when they are fully seasoned. The berries of *A. unedo* are rarely consumed as fresh fruit, but have some importance in farming communities where they are used in the production of alcoholic beverages, jams, jellies and marmalades [327, 328]. In folk medicine the fruits are also used as antiseptics, diuretics and laxatives, while the leaves have been reported as possessing several biological properties, it has been used as astringent, diuretic, urinary antiseptic agent, anti-inflammatory [329], anti-diarrheal [330] and, more recently, it presented potential in the treatment of hypertension [260,] cardio vascular diseases [331, 332] and diabetes [333, 334].

A. unedo leaves are rich in phenolic compounds, including tannins, flavonoids, phenolic glycosides, among others, and α-tocopherol. The fruits contain several antioxidant molecules, phenolic compounds (e.g. anthocyanins, gallic acid derivatives, tannins and flavonoids), vitamin C, vitamin E and carotenoids [327, 328, 335-338].

Taking into account the folklore of *A. unedo*, its phytochemical content and in particular its antioxidant activity, it is of considerable interest to investigate its potential application as a therapeutic agent in a wide range of human diseases, particularly in neurodegenerative disorders [335, 339].

Figure 8: *Corema album* (left) and *Arbutus unedo* (right).

4. Aim

This work focused on the study of Portuguese plants as sources of (poly)phenols with neuroprotective potential in PD, and explore their mechanisms of action. To achieve this goal intermediary aims were determined:

- Exploit the molecular determinants of aSyn toxicity and gain insight into the regulation of aSyn clearance by phosphorylation in a yeast model of PD.

- Explore the bioactivities of (poly)phenols through a pharmacological approach. *C. album* leaves (poly)phenols were tested in yeast and H4 cell models of PD to assess their effects and mechanisms of action. *In vitro* assays were also used to understand the effect of (poly)phenols in the fibrillization of aSyn.

- Gain insight into nutritional-related mechanisms of action of (poly)phenols. *A. unedo* (poly)phenols were submitted to an *in vitro* gastrointestinal digestion procedure. Its neuroprotective potential was assessed in cellular models of PD, and the protection mechanisms of dietary (poly)phenols were assessed.

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Chapter 1:

Blocking alpha-synuclein phosphorylation impairs its clearance through autophagy

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This chapter is based on the following manuscript:

Phosphorylation modulates clearance of alpha-synuclein inclusions in a yeast model of Parkinson's disease

Tenreiro S, Reimão-Pinto M, Antas P, Rino J, Wawrzycka D, Macedo D, Rosado-Ramos R, Amen T, Waiss M, Magalhães F, Gomes A, Santos CN, Kaganovich D, Outeiro TF. *PLOS Genetics*, 2014 10(5): e1004302.

This chapter contains data in which the author of this dissertation executed the majority of the experiments. Figure 1B was performed by Gomes A and figure 4B by Reimão-Pinto M.

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Abstract

Alpha-synuclein (aSyn) is the main component of proteinaceous inclusions known as Lewy bodies (LBs), the typical pathological hallmark of Parkinson's disease (PD) and other synucleinopathies. Although aSyn is phosphorylated at low levels under physiological conditions, it is estimated that ~90% of aSyn in LBs is phosphorylated on S129 (pS129). Nevertheless, the significance of pS129 in the biology of aSyn and in PD pathogenesis is still controversial.

Here, we harnessed the power of the budding yeast in order to assess the implications of phosphorylation on aSyn cytotoxicity, aggregation and sub-cellular distribution. We found that aSyn is phosphorylated on S129 by yeast endogenous kinases. Interestingly, phosphorylation reduced aSyn toxicity and the percentage of cells with cytosolic inclusions, in comparison to cells expressing the mutant form of aSyn (S129A) that mimics the unphosphorylated state of aSyn.

Upon blockade of aSyn expression, cells were able to clear the inclusions formed by WT aSyn. However, this process was much slower for the inclusions formed by S129A aSyn. Interestingly, whereas the accumulation of WT aSyn led to a marked induction of autophagy, cells expressing the S129A mutant failed to activate this quality control pathway. The finding that the phosphorylation of aSyn on S129 can alter the ability of cells to clear aSyn inclusions, provides important insight into the role that this posttranslational modification may have in the pathogenesis of PD and other synucleinopathies. Ultimately, opening novel avenues for investigating the molecular basis of these disorders and for the development of therapeutic strategies.

Introduction

Protein misfolding and aggregation is an unavoidable and widespread problem in biology. Cells evolved a series of quality control mechanisms to ensure overall proteostasis and, in some cases, to exploit the plasticity of diverse conformational states, including those concealed in protein aggregates, as in the case of certain types of prions. In other instances, protein aggregates can be detrimental [1, 2]. Protein inclusions made of alpha-Synuclein (aSyn), known as Lewy bodies (LBs) are the pathological hallmark of Parkinson's disease (PD) and other disorders known as synucleinopathies [3, 4]. The normal function of aSyn is still unclear, but it is thought to be involved in the regulation of dopamine neurotransmission, vesicular trafficking and in synaptic function and plasticity [5, 6]. Although aSyn is phosphorylated at low levels under physiological conditions, a striking 90% of aSyn is phosphorylated on S129 (pS129) in LBs [7]. However, the significance of pS129 in the pathogenesis of synucleinopathies is unresolved. While studies in *Drosophila* [8] and transgenic mouse models of PD [9] showed that pS129 aSyn was pathogenic, studies in rats and in *C. elegans* failed to associate toxicity with phosphorylation, and suggested a role of pS129 in the attenuation of aSyn induced neuronal dysfunction [10, 11]. However, no differences in toxicity or aggregate formation were seen in a rat model [12]. Whether pS129 promotes or prevents aggregation remains largely controversial [13-15].

The yeast *Saccharomyces cerevisiae* is a powerful model for the study of protein misfolding due to the high conservation of the quality control systems with all other eukaryotes, including humans[16]. Although *S. cerevisiae* lacks an aSyn ortholog, heterologous expression of the protein induces toxicity in a concentration dependent manner and is associated with the formation of cytoplasmic protein inclusions[17]. Moreover, a network of highly conserved aSyn interactors was identified, suggesting the protein can be studied using simple models such as yeast, worms, or flies, in addition to mammalian models [18, 19]. Several pathways involved in aSyn-associated toxicity in yeast are conserved in other eukaryotic models of PD. This is the case of apoptosis [17], lipid droplet accumulation [17] mitochondrial dysfunction [20, 21], proteasome impairment [17, 22, 23], oxidative stress [22, 24], autophagy and mitophagy dysfunction [25, 26], vesicle trafficking defects [17, 27], and ER-to-Golgi

trafficking impairment [21, 28, 29].

PD pathogenesis is thought to be exacerbated from inefficient protein clearance as consequence of dysfunction in protein degradation [30, 31]. Clearance of aSyn can occur through direct proteolysis [32], the ubiquitin-proteasome system (UPS) [33] and/or chaperone-mediated autophagy (CMA) [34]. However, under pathological conditions, aSyn inhibits the proteasome [23, 35] and impairs CMA, leading to the upregulation of macroautophagy (hereafter referred as autophagy) [36, 37]. Autophagy dysfunction plays a central role in PD [25, 36, 38] and was shown to be required for aSyn degradation under pathological conditions [39]. Accordingly, increasing evidence suggests the existence of a complex cross-talk between different forms of autophagy and also between autophagy and the proteasomal degradation pathway, processes known to play distinct roles in the clearance of specific species of aggregated aSyn [23, 35, 36, 40, 41].

In yeast, the clearance of aSyn inclusions has been associated with both UPS and autophagocytic degradation pathways [42]. However, recent observations suggest the UPS may be less relevant in mediating aSyn clearance [26].

Here, we explored the power of yeast genetics in order to gain mechanistic insights into the role of S129 phosphorylation on aSyn biology. Using the S129A mutant of aSyn, that mimics the unphosphorylated state of aSyn, we found that blocking phosphorylation increases aSyn toxicity and promotes the formation of cytosolic inclusions. Our data are consistent with the involvement of phosphorylation in the clearance of aSyn via autophagy.

Altogether, our study provides novel insight into the role of S129 aSyn phosphorylation and opens novel avenues for additional studies in higher model systems.

Results

Blockade of aSyn S129 phosphorylation promotes toxicity and aggregation

To investigate the effect of aSyn phosphorylation in yeast, we used strains carrying two copies of human *SNCA* cDNA integrated in the genome. Encoding either wild-type (WT) aSyn or the S129A mutant, in order to block phosphorylation. Both constructs are fused to GFP (aSyn-GFP) under the regulation of a galactose inducible promoter (*GAL1*). These strains were previously described and characterized [43] and display a moderate level of aSyn toxicity compared to strains described in other studies [17, 28, 29]. Viability of the different strains were assessed using propidium iodide (PI), a dye that stains cells whose membrane integrity was compromised (Fig. 1A). After 6 h of aSyn expression induction, 13.6 ± 0.2% of the yeast cells expressing S129A aSyn-GFP were PI positive, compared to only 2.1 ± 0.2% of cells expressing WT aSyn-GFP (Fig. 1A). These results demonstrated that expression of S129A aSyn is more toxic for yeast cells than expression of WT aSyn.

We then assessed the correlation between cytotoxicity and the aggregation of aSyn-GFP. It was previously described that initially aSyn associates with the plasma membrane [17]. Upon increased accumulation of the protein, inclusions appear adjacent to the plasma membrane and, finally, become cytoplasmic inclusions [17, 28, 42].

In order to determine the effect of phosphorylation on aSyn aggregation, WT and S129A aSyn-GFP were submitted to size exclusion chromatography (SEC), a widely established method for the biochemical characterization of protein species. In the conditions of this procedure the protein aggregates maintain their oligomerization state and do not disaggregate. For WT aSyn-GFP, we detected species in fractions corresponding to molecular weights of 440 kDa (Fig. 1B). Interestingly, for S129A aSyn-GFP, we detected species in fractions corresponding to even higher molecular weights, since these eluted in the void volume of the column (Fig. 1B).

By using an antibody that specifically recognizes pS129-aSyn, we observed that human aSyn was phosphorylated on S129 residue by endogenous kinases as assessed by western-blot (data not shown and Fig. S1 A).

pS129-aSyn aggregation was also analysed using an antibody that specifically recognizes aSyn phosphorylation at this residue. pS129-aSyn showed a similar distribution to that of WT aSyn-GFP (Fig. 1B). This result convincingly demonstrates, for the first time, that in the yeast model considerable large oligomeric species of aSyn-GFP are formed, an issue that had not been previously resolved in the field.

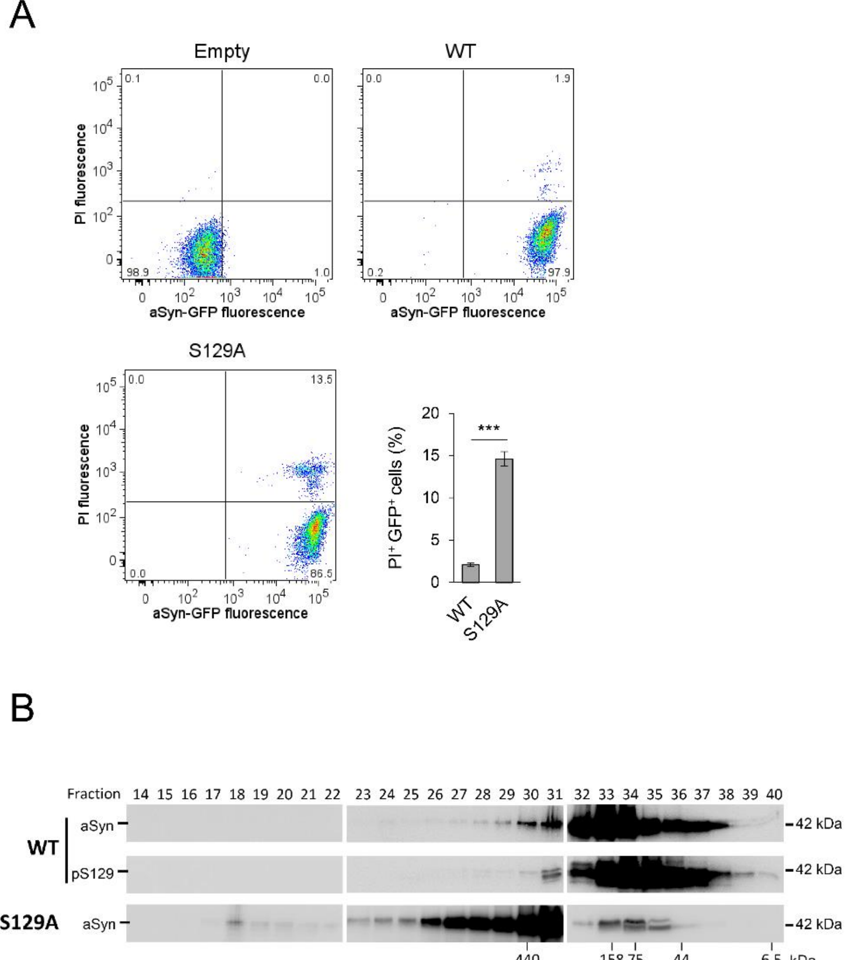


FIGURE 1. S129A aSyn is more toxic and forms oligomeric species with higher molecular weight than the WT aSyn. A) aSyn-GFP vs. Propidium Iodide (PI) fluorescence and percentage of PI and GFP positive cells assessed by flow cytometry, in the indicated yeast cells, after 6 h of aSyn expression induction. B) The aggregated species formed by WT and S129A aSyn-GFP were resolved using size exclusion-chromatography (SEC) after 6 h of aSyn-GFP expression induction. Fractions were collected and separated on SDS-PAGE followed by western blot analysis with an antibody against aSyn or pS129 aSyn. The molecular weights indicated correspond to the calibration curve

(results not shown) performed in the SEC. A representative result is shown and values represent the mean ± SD of at least three independent biological experiments.***$p<0.001$.

We excluded whether the differences in toxicity and aggregation were due to different expression levels of aSyn-GFP. Using western blot analyses, we found that WT and S129A aSyn-GFP were expressed at similar levels after 6 h of expression induction (data not shown). Altogether, blocking phosphorylation on the S129 residue led to a marked increase in aSyn toxicity and to the formation of larger inclusions (Fig. 1).

Modulation of aSyn S129A toxicity by modifiers of ER-to-Golgi trafficking

Several studies demonstrated that aSyn may disrupt multiple intracellular trafficking pathways in yeast [17, 18, 21, 27-29, 43, 44]. However, the endoplasmic reticulum (ER)-to-Golgi vesicular trafficking impairment is one of the first defects following induction of aSyn expression [29]. This defect can be rescued by genes promoting ER-to-Golgi trafficking (*YPT1*, *YKT6*, *UBP3*, and *BRE5*). In addition, genes negatively regulating ER-to-Golgi trafficking (*GYP8* and *PMR1*) enhance aSyn toxicity [29]. To determine if the increased toxicity observed by the blockade of aSyn phosphorylation was also associated with ER-to-Golgi trafficking defects, we tested the effects of previously described suppressors (*YPT1, YKT6, UBP3*, and *BRE5*) and enhancers (*GYP8* and *PMR1*) of aSyn toxicity in yeast [29]. These modifiers of aSyn toxicity were co-expressed with either WT or S129A aSyn-GFP and the effect on toxicity and inclusion formation was evaluated 6 h after induction (Fig. 2 and 3).

Expression of the ER-to-Golgi trafficking enhancers *YPT1* and *UBP3* significantly reduced the toxicity of S129A aSyn-GFP (Fig. 2), while the overexpression of *BRE5* and *YPT1* was not able to rescue S129A aSyn-GFP toxicity (Fig. 2). Regarding the two enhancers of aSyn toxicity, *GYP8* and *PMR1*, expression of an extra copy of these genes exacerbated WT aSyn toxicity as expected [29], as well as S129A aSyn toxicity.

The formation of aSyn-GFP fluorescent inclusions were also assessed. The S129A mutant presented more inclusions than the WT aSyn (Fig. 3A), in agreement with the increased toxicity and inclusions size of S129A (Fig. 1 and results not shown). Expression of *YPT1, YKT6* and *UBP3* significantly reduced the formation of both WT and S129A aSyn-

GFP inclusions (Fig. 3A) while the overexpression of Bre5 only decreased the formation of WT aSyn-GFP inclusions, and had no effect on S129A inclusion formation (Fig. 3A).

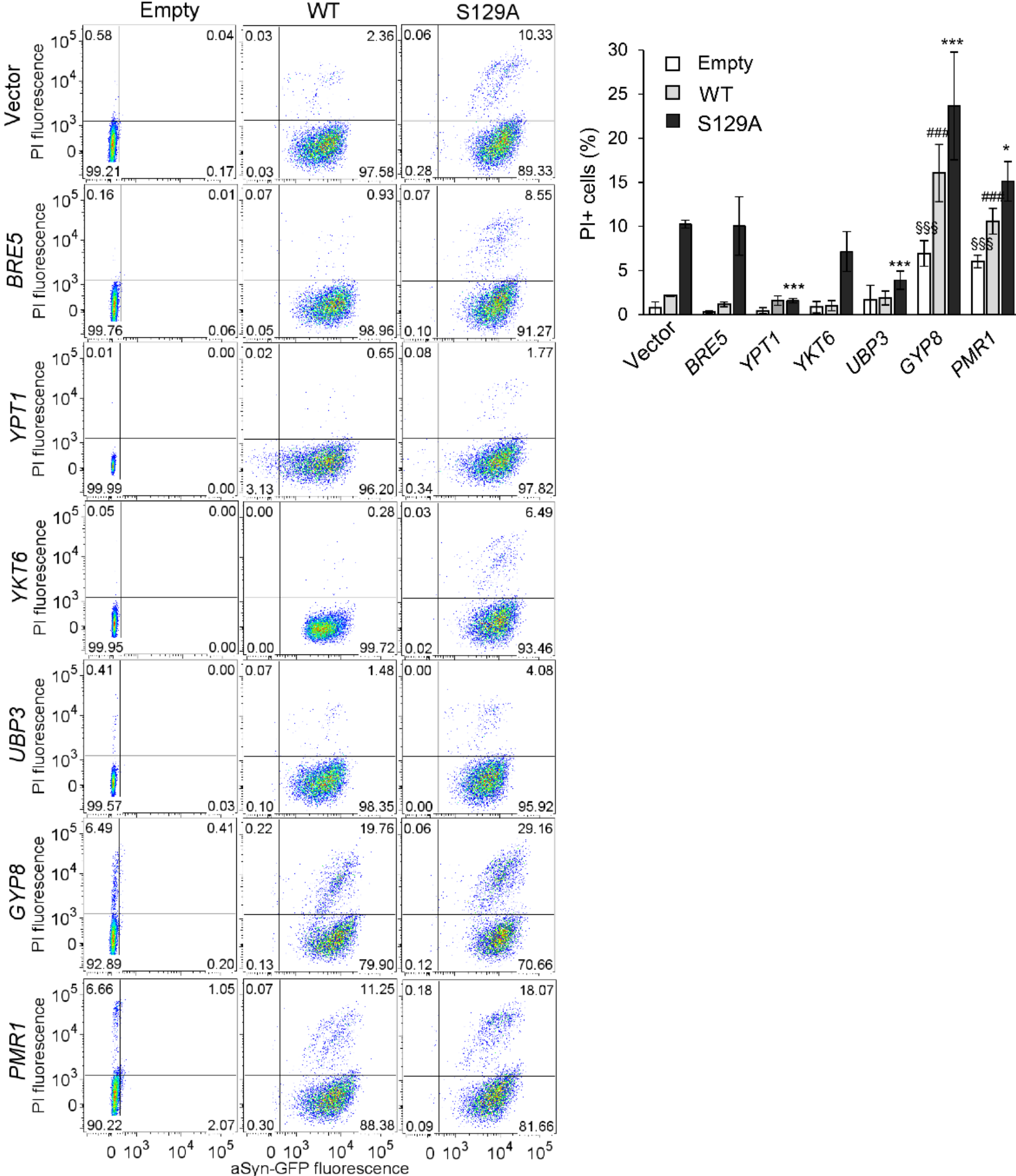


FIGURE 2. ER-to-Golgi trafficking genes are modifiers of S129A aSyn toxicity. aSyn-GFP vs. Propidium Iodide (PI) fluorescence and percentage of PI positive cells assessed by flow cytometry 6 h after aSyn expression induction. A representative result is shown and values represent the mean ± SD of at least three independent biological experiments. §§§ p<0.001 *vs* empty strain; *** p <0.001 or *p<0.05 *vs* WT; ### <0.001 or #p<0.05 *vs* S129A.

Regarding the two enhancers of aSyn toxicity, *GYP8* and *PMR1*, expression of an extra copy of these genes exacerbated WT aSyn toxicity as expected [29], and had a similar effect on S129A aSyn toxicity (Fig. 2). *GYP8* and *PMR1* also increased the formation of aSyn inclusions in WT aSyn-GFP expressing cells without affecting the percentage of cells with S129A aSyn-GFP inclusions (Fig. 3A).

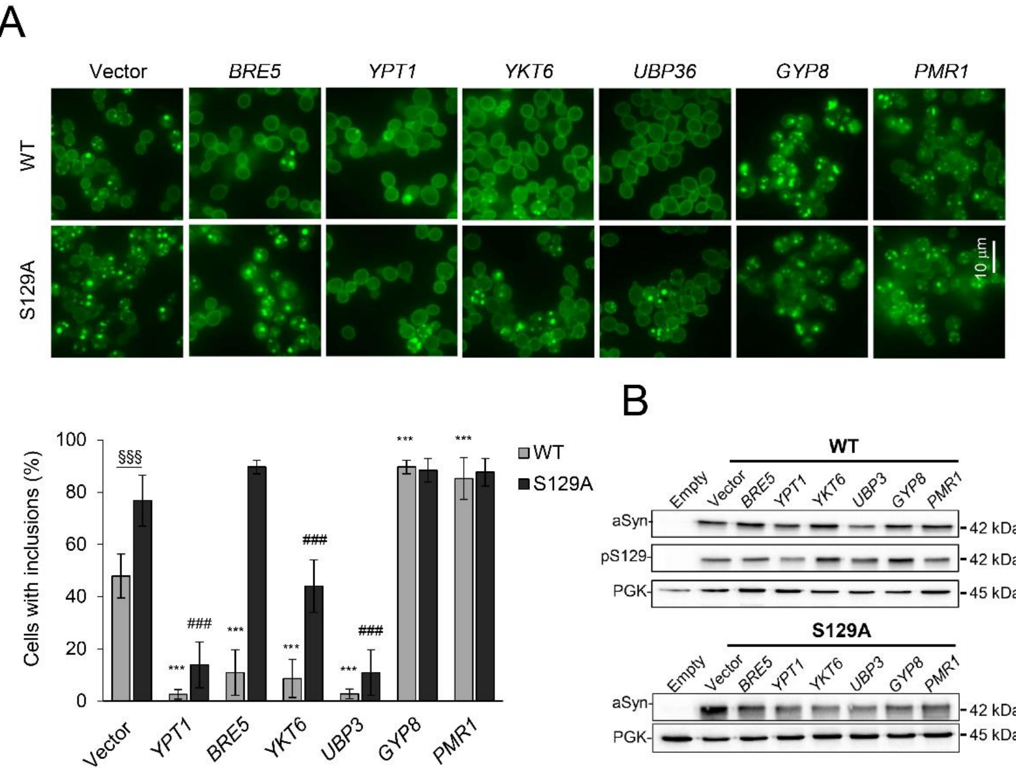


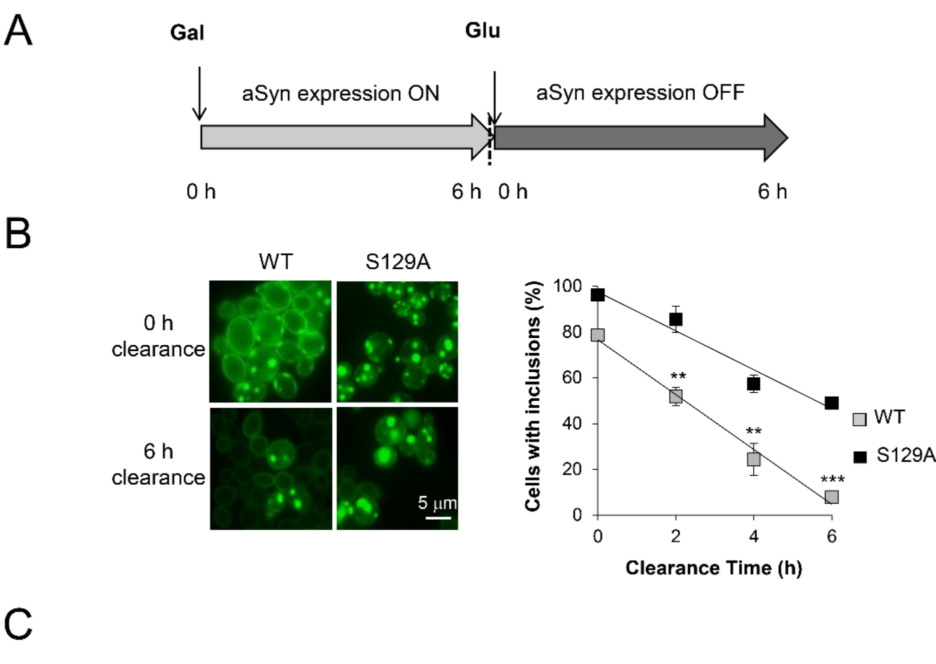
FIGURE 3. Modulation of ER-to-Golgi trafficking alters S129A aSyn inclusion formation. A) Intracellular localization of the WT or S129A aSyn-GFP (higher panel) and percentage of yeast cells containing aSyn-GFP inclusions (lower panel) in the indicated yeast strains, assessed by fluorescence microscopy after 6 h of aSyn-GFP expression induction. **B)** WT or S129A aSyn-GFP expression and pS129 levels in the indicated yeast strains assessed by western blot analysis of total protein extracts 6 h after aSyn-GFP expression induction. A representative result is shown and values represent the mean ± SD of at least three independent biological experiments. §§§ p <0.001 between the indicated strains; *** p <0.001 *vs* WT; [###] <0.001 *vs* S129A.

To determine if the phenotypes observed resulted from an effect of the modifiers on the protein levels of WT and S129A aSyn-GFP, we preformed western blot analyses. We found that total WT and S129A aSyn-GFPlevels were not affected by the co-expression of the modifiers (Fig 3B). Moreover, the pS129 levels of the WT aSyn –GFP were also not altered by the co-expression of the modifiers (Fig 3B). Altogether, these results indicate that ER-Golgi vesicle trafficking defects observed for S129A aSyn, can also be rescued by genes involved in the suppression of WT aSyn toxicity.

Blockade of aSyn phosphorylation compromises its degradation

We postulated that the different toxicity and aggregation of S129A aSyn-GFP might arise from differences in its protein clearance. To test this, aSyn expression was stopped after 6 h by replacing galactose by glucose (to repress the *GAL1* promoter), afterwards aSyn-GFP clearance was followed for 6 h (Fig. 4A). We found that 49 ± 0.4% of cells expressing S129A aSyn-GFP contained inclusions, while only 8 ± 1.2% of the ones expressing the WT form presented aSyn-GFP inclusions, after the clearance period (Fig. 4B). This corresponds to a reduction of about 50% and 70% when compared to the initial percentage of cells with inclusions, respectively (Fig. 4B).

To further investigate how phosphorylation affected aSyn inclusion formation and its characteristics, cells were analysed by flow cytometry (Fig. 4C). At 0 h of clearance, both strains presented a homogeneous distribution of the GFP fluorescence showing a single population of cells (Fig. 4C). However, after 6 h of clearance, two populations were visible for both WT and S129A aSyn-GFP, one with weaker and other with stronger GFP fluorescence (Fig. 4C). We found that the strain expressing S129A aSyn-GFP accumulated more cells in the population displaying stronger GFP signal (11 ± 3.5% vs. 1.4 ± 1.0% for WT aSyn-GFP). Together with the results from the microscopy (Fig. 4B) and SEC (Fig. 1B), these findings are consistent with cells accumulating inclusions of different sizes, with the larger inclusions displaying stronger GFP signal (Fig. 4B and C).



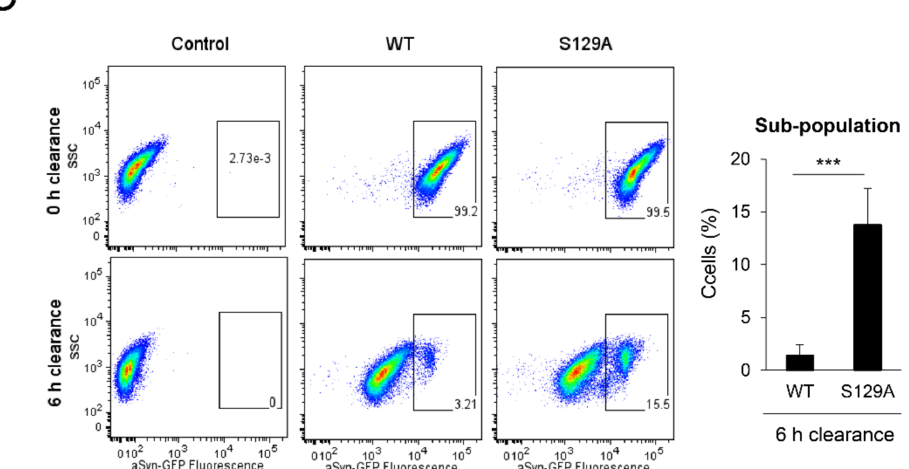


Figure 4. The clearance of S129A aSyn inclusions is compromised. A) Schematic representation of the treatments performed: aSyn expression was induced for 6 h in galactose medium (Gal) and then the carbon source was changed to glucose (Glu) to repress aSyn expression for 6 h. B) Intracellular localization of WT or S129A aSyn-GFP and percentage of yeast cells containing aSyn-GFP inclusions, assessed by fluorescence microscopy. C) SSC (side scatter) and aSyn-GFP fluorescence assessed by flow cytometry, at 0 and 6 h after aSyn clearance. A sub-population of yeast cells presenting higher levels of GFP fluorescence is distinguishable after 6 h of WT or S129A aSyn clearance (left panel, gated cells). The percentage of this sub-population is represented in the right panel. A representative result is shown and values represent the mean ± SD of at least three independent biological experiments. ***p<0.001, **p<0.01.

Unphosphorylatable aSyn fails to induce autophagy

To gain further insight into the proteostasis of aSyn we analysed the contribution of autophagy to the clearance of WT and S129A aSyn-GFP. We used a mCherry-Atg8 and inserted the reporter under the control of the endogenous ATG8 promoter, in the genome of the strains expressing WT or S129A aSyn-GFP (Fig. 5)

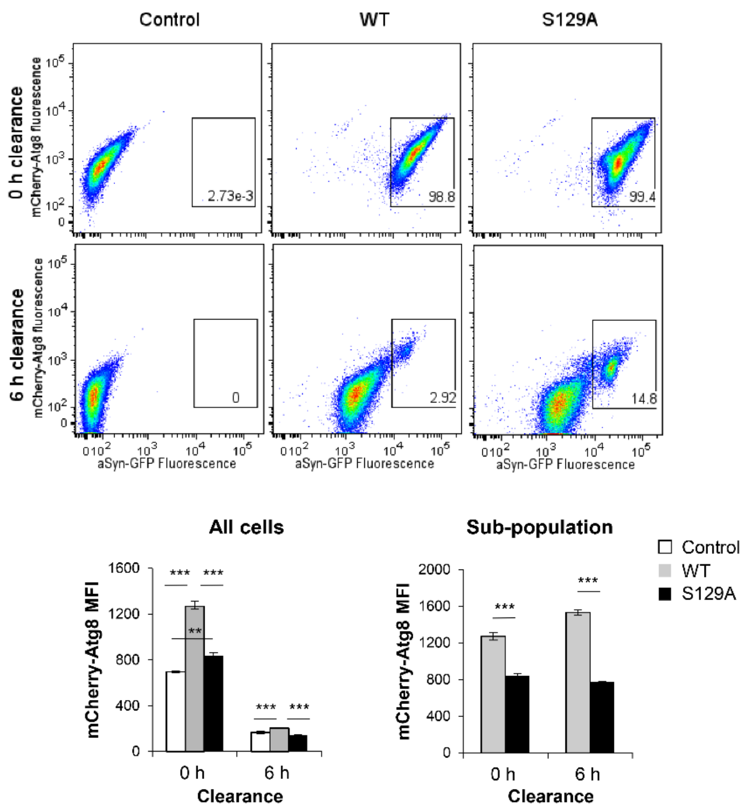
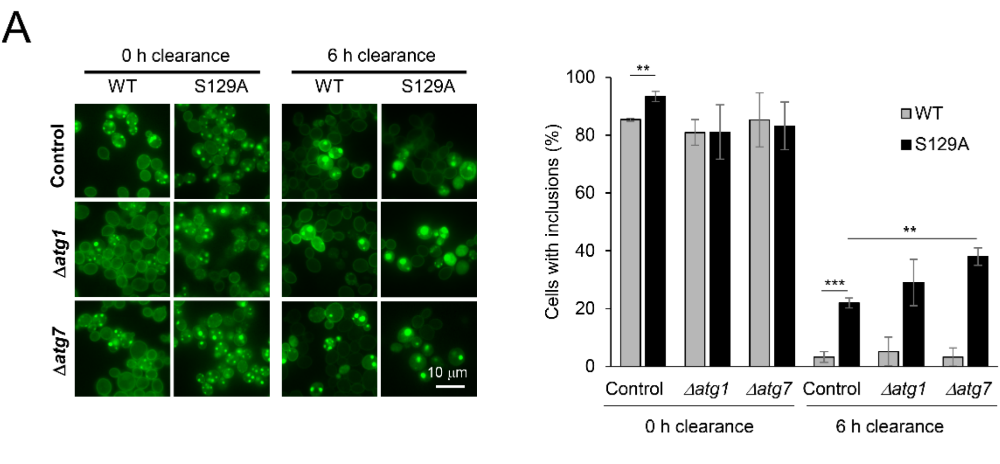


FIGURE 5. S129A aSyn impairs Atg8 induction. A) aSyn-GFP and mCherry-Atg8 fluorescence intensity in yeast cells co-expressing mCherry-Atg8 with the empty vector (control) or with either WT or S129A aSyn-GFP, at the indicated time points, assessed by flow cytometry (upper panel). mCherry-Atg8 MFI from all cells (lower left panel) and from the indicated sub-population (lower right panel). A representative result is shown and values represent the mean ± SD of at least three independent biological experiments. ***p <0.001.

Using flow cytometry, we established a correlation between autophagy induction (measured by mCherry fluorescence intensity) and aSyn-GFP signal (Fig. 5). At 0 h of

clearance the mCherry-Atg8 median fluorescence intensity (MFI) in cells expressing S129A aSyn-GFP were considerably lower than those in cells expressing WT aSyn, and decreased gradually during the clearance period to near basal levels (Fig. 5). As expected, it was visible that, during clearance, the fluorescence of either WT or S129A aSyn-GFP decreased (Fig. 5). However, in both cases a sub-population of cells with larger and brighter inclusions maintained stronger GFP fluorescence after 6 h of clearance, in agreement with the clearance results in Fig. 4. In this sub-population, higher levels of mCherry-Atg8 were observed both in the cells expressing WT or S129A aSyn-GFP, indicating that autophagy induction is more pronounced in cells with bigger and brighter aSyn inclusions (Fig. 5). However, in this sub-population, the cells expressing S129A aSyn-GFP displayed lower levels of mCherry-Atg8 than the cells expressing WT aSyn-GFP both at 0 and 6 h of clearance (Fig. 5). Indicating a deficit in autophagy induction on the S129A variant, concomitant with the increase in inclusions and toxicity (Fig. 1 and 4).

A second line of evidence for the effect of pS129 on aSyn clearance by autophagy was obtained by genetically modulating this pathway. For these studies, we deleted *ATG1* and *ATG7* genes in the WT and S129A aSyn-expressing strains. Atg1 is a kinase playing an important role in autophagy initiation [45] and its mutant is defective in autophagy [46] while Atg7 is an activator of Atg8 and is required for the formation of autophagic bodies [47]. Deletion of *ATG1* and *ATG7* significantly increased the percentage of cells displaying aSyn inclusions after 6 h of clearance (Fig. 6A). Flow cytometry experiments confirmed the fluorescence microscopy results, showing a significant increase in the population of cells displaying stronger GFP signal when autophagy was impaired due to *ATG1* or *ATG7* deletion (Fig. 6B). Deletion of *ATG1* and *ATG7* did not significantly affect WT or S129A aSyn-GFP expression levels after 6 h of induction (Fig. 7A). However, when aSyn expression was turned off and clearance was followed during 18 h, S129A presented an accumulation of aSyn. Moreover, Δ*atg7* resulted in higher levels of WT and S129A aSyn-GFP, an effect that was more pronounced with the phosphorylation-resistant mutant (Fig. 7A). Deletion of *ATG1* and *ATG7* did not significantly affect the pS129 in levels in the aSyn WT strain, either after 0 or 6 h of clearance (Fig. S1 B).



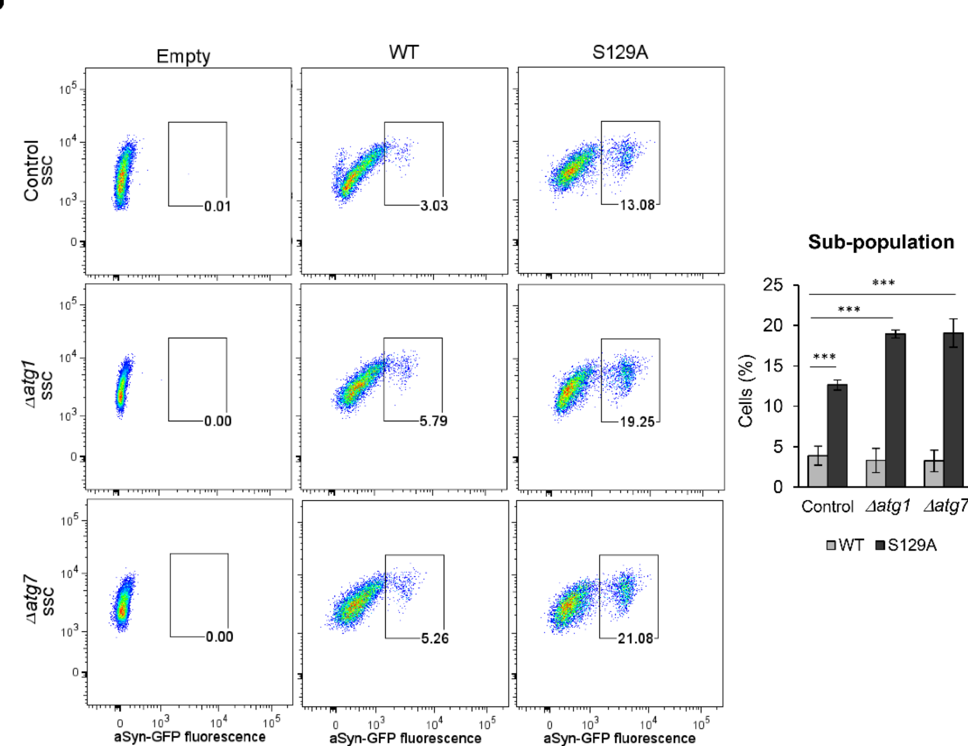


FIGURE 6. Impairment of autophagy decreases S129A aSyn-GFP clearance. A) Intracellular localization of WT or S129A aSyn-GFP (left panel) and percentage of cells containing aSyn-GFP inclusions, assessed by fluorescence microscopy (right panel). **B)** Side scatter (SSC) vs. aSyn-GFP fluorescence assessed by flow cytometry, at 6 h of aSyn clearance (left panel) and percentage of cells in the sub-population of cells presenting higher levels of GFP fluorescence (right panel). A representative result is shown and values represent the mean ± SD of at least three independent biological experiments. ***p <0.001; **p<0.01, *p<0.05

Moreover, Δ*atg1* and Δ*atg7* S129A aSyn-GFP expressing cells also exhibited a higher percentage of PI positive cells, indicating that impairment of autophagy increased S129A aSyn-GFP toxicity, an effect that was not observed for WT aSyn-GFP expressing cells (Fig. 7B). Altogether these results indicate that blocking aSyn phosphorylation has a negative impact on autophagy induction, suggesting that this post-translational modification will lead to a different fate of aSyn in cells.

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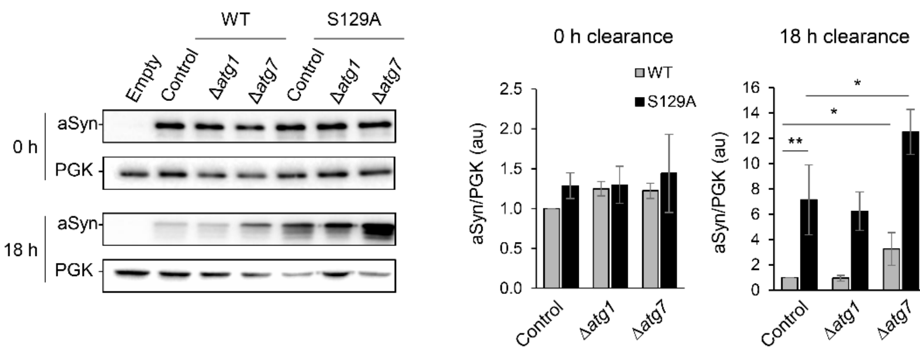

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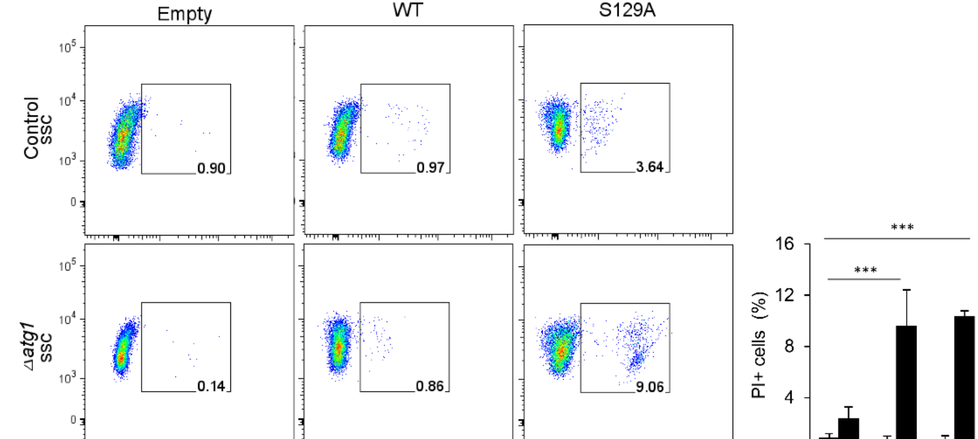

FIGURE 7. Impairment of autophagy decreases S129A aSyn-GFP clearance and increased its toxicity. A) WT and S129A aSyn-GFP expression levels assessed by western blot analysis of total protein extracts at the indicated time points of aSyn-GFP clearance (left panel). Densitometric analysis of the immunodetection of aSyn normalized to PGK levels, presented in arbitrary units (a.u.) (right panel). **B)** Side scatter (SSC) vs. propidium iodide (PI) fluorescence (left panel) and percentage of PI positive cells (right panel), assessed by flow cytometry after 6 h of aSyn clearance. A representative result is shown and values represent the mean ± SD of at least three independent biological experiments ***p <0.001; **p<0.01, *p<0.05.

Discussion

In this work we found that WT aSyn-GFP is strongly phosphorylated at S129 by endogenous yeast kinases. The S129A mutation was found to promote aSyn fibrillization [11, 48]. However, this was not consensual in all cell and animal models where S129A aSyn was expressed. In SH-SY5Y cells, a neuroblastoma cell line with dopaminergic characteristics, S129A aSyn expression reduces inclusion formation [13], while in a *Drosophila* model, S129A aSyn expression results in the accumulation of increased levels of aSyn oligomers, but not of mature fibrils [8]. In our yeast model, expression of the phosphorylation-deficient S129A aSyn-GFP resulted in an exacerbation of aSyn toxicity concomitantly with a reduction in cellular viability and an increase in aSyn inclusion formation.

Despite the accumulating evidence favouring the hypothesis that soluble aSyn oligomers, rather than insoluble protein aggregates, are the cytotoxic species in PD, the question is still unresolved [49, 50]. In yeast, aSyn inclusions were described as clusters of vesicles [27, 28, 43], raising questions about whether aSyn accumulations actually displayed biochemical properties compatible with the formation of protein aggregates. However, at least some of these accumulations are indeed amyloid-like and β-sheeted aggregates, as they react with thioflavin S [42] or thioflavin T [51]. Here, we biochemically characterized the aSyn-GFP species that are formed in yeast cells using size exclusion chromatography and clearly demonstrate the formation of large oligomeric species. Moreover, we established a correlation between increased inclusions formation and exacerbation of cytotoxicity and the formation of oligomeric species with higher molecular weight for the S129A aSyn-GFP mutant. These results are consistent with other reports where large aSyn soluble oligomers are also considered to constitute the toxic species [52].

Among the various cellular defects that have been implicated in the etiology of synucleinopathies, vesicular trafficking impairment has emerged as a major component of aSyn-dependent toxicity in yeast and in other model organisms [17, 18, 21, 27-29, 43, 44]. Both genetic and chemical modulation of vesicular trafficking modulate aSyn toxicity [18, 21, 29]. In this study we show that pS129 blockade exacerbates vesicular trafficking defects that can be relieved by overexpression of *YPT1*, *YKT6* and *UBP3*, genes that increase the forward transport

between ER and Golgi. Ypt1, the Rab guanosine triphosphatase whose mammalian orthologue Rab1 is able to prevent dopaminergic neuron loss [29], plays an essential role in the tethering and docking of the transport vesicle with the Golgi [53]. *YKT6*, the soluble NSF (N-ethylmaleimide–sensitive factor) attachment protein receptor (SNARE), increases forward transport by increasing the likelihood of membrane vesicles from the ER tethering to Golgi target membranes [54]. In turn, the ubiquitin protease Ubp3, together with its cofactor Bre5, function to deubiquitinate the COPII coat protein Sec23p, and likewise promote vesicle exit from the ER [55]. Interestingly, the Bre5 cofactor, which also suppresses aSyn toxicity [29], was not able to restore S129A aSyn-GFP induced trafficking defect. This suggests that aSyn phosphorylation may modulate the way the protein interacts with components of the trafficking pathway, as one might expect.

Moreover, *GYP8* and *PMR1* whose overproduction negatively regulates ER-Golgi trafficking, exacerbate S129A aSyn-GFP toxicity. Gyp8, is a negative regulator of Ypt1, that therefore inhibits ER-to-Golgi trafficking [56], while Pmr1 is the major Golgi membrane P-type ATPase ion pump responsible for transporting Ca^{2+} and Mn^{2+} ions into the Golgi apparatus ions important for proper processing and trafficking of proteins through the secretory pathway [57].

Proteostasis is a central concept in the context of several disorders [58]. An imbalance between the rates of protein synthesis, clearance, and aggregation, caused by proteostasis dysfunction, could favour accumulation and/or formation of protein oligomers and inclusions that contribute to cytotoxicity [59]. We found that blocking aSyn phosphorylation impaired the turnover of aSyn.

When the synthesis of aggregation-prone proteins surpasses the degradation capacity of the cell, different quality-control mechanisms, conserved from yeast to mammalian cells, actively sequester aggregated proteins as a protective cellular response[50].

Post-translation modifications modulate the degradation of aggregate-prone proteins by the UPS and/or autophagy. Ubiquitination generally determines whether a protein is degraded via the UPS or autophagy [60]. Interestingly, phosphorylation of mutant huntingtin (Htt), the protein associated with Huntington's disease, was found to precede and regulate additional post-translational modifications, including ubiquitination, SUMOylation, and

acetylation, enhancing its normal clearance by the proteasome and lysosome [61]. In particular, acetylation of mutant huntingtin promotes its targeting to autophagosomes, facilitating its specific degradation by the autophagy/lysosomal pathway [62].

The impact of aSyn S129 phosphorylation on its clearance only started to be investigated recently. In neuronal cell lines, it was observed that proteasome inhibition results in increased levels of pS129 aSyn as an outcome of either increased activity of the kinase(s) involved or decreased phosphatase activity, together with decreased degradation of pS129 aSyn by the proteasome in a ubiquitin-independent manner [63, 64].

Recently, overexpression of GRK6, one of the kinases capable of phosphorylating aSyn on S129 [65], was found to moderately increase aSyn toxicity in a rat model of familial PD [66]. In contrast, another recent study showed that the overexpression of another kinase, the Polo-like kinase 2 (PLK2) [67], is protective by mediating selective autophagy clearance of pS129 aSyn [68]. This apparent discrepancy could be the reflex of the different efficiencies of these kinases to phosphorylate aSyn on S129. In our yeast model, we completely abolished aSyn phosphorylation by replacing S129 with a phosphorylation-resistant amino acid (alanine). In fact, our results are consistent with those recently reported [68], since we observed that blocking aSyn phosphorylation compromises its degradation. The clearance of the inclusions formed by S129A aSyn was slower than that of inclusions formed by WT aSyn. Moreover, our findings suggest that autophagy is the main mechanism involved in aSyn clearance in our yeast PD model. Whereas the accumulation of WT aSyn led to a marked induction of autophagy, cells expressing the S129A mutant failed to activate this pathway. Thus, we postulate that S129 phosphorylation might constitute a switch to sense and induce the autophagocytic pathway, and that blocking phosphorylation impairs autophagic induction, albeit without altering the autophagic flux (data not shown) [69].

Genetic impairment of yeast autophagy by deletion of *ATG1* and *ATG7* did not significantly affect the levels of WT or S129A after 6 h of induction suggesting that, at this time point, clearance by autophagy is not superimposed to protein synthesis or that cells could compensate autophagy impairment through other clearance pathways, as suggested by studies performed in other cellular models [39, 40, 70]. However, *ATG7* deletion increased the defect on the clearance of S129A aSyn-GFP, but had no significant effect on WT aSyn-GFP

clearance. This effect could be due to the accumulation of different aSyn species formed by S129A or WT aSyn, as we clearly demonstrated in this study. While soluble and smaller oligomeric species of aSyn could be more easily cleared by the proteasome and CMA, as reported in other models [33, 34, 71, 72], the larger oligomeric species formed by S129A aSyn-GFP could specifically require autophagy function for clearance. Our observation that aSyn accumulation leads to impairment of proteasome function [73], as previously observed [74], is also consistent with this hypothesis. However, it is now evident that autophagy and proteasome function are deeply interconnected and that inhibition of either one of these pathways results in the compensatory upregulation of the other [39, 70]. Thus, we postulate that upon aSyn-mediated proteasome impairment, autophagy is upregulated as a compensatory mechanism to deal with the excess of aSyn.

In this study, we provide evidence supporting a novel link between aSyn phosphorylation, aggregation and cellular toxicity using a simple but powerful model organism. The finding that the phosphorylation state of aSyn on S129 can have an impact in the ability for cells to clear aSyn inclusions opens novel venues for intervention in synucleinopathies through the modulation of aSyn phosphorylation.

Materials and methods

Plasmid, yeast strains and culture conditions

The yeast strains and plasmids used in this work are described in [73] and were constructed by Dr. Sandra Tenreiro, Instituto de Medicina Molecular, Portugal. VSY71 to VSY73 contain double genome insertions of *GAL1pr-SNCA*(WT, S129A)-GFP or of the empty vectors and were previously described [43]. Yeast transformations were carried out using a standard lithium acetate procedure [75].

For aSyn expression induction experiments, yeast cells were pre-grown in YEP-Raffinose (peptone 2%, yeast extract 1%, raffinose 1%) liquid media at 30 °C, with orbital agitation (200 rpm) for 24 h (doubling time: ~3 h). After 24 h, optical density at 600 nm ($OD_{600\ nm}$) was measured and yeast cells were diluted to a standardized $OD_{600\ nm}$ = $3x10^{-3}$ (~$2.5x10^{5}$ cells/mL) in YEP-Raffinose liquid media (no repression of the galactose-inducible promoter) and grown at 30 °C, with orbital agitation (200 rpm). After 24 h, $OD_{600\ nm}$ was measured. The volume of yeast culture needed to inoculate a new culture with an initial standardized $OD_{600\ nm}$ = 0.2 (~$7x10^{6}$ cells/mL) was centrifuged (3000 rpm, at 30 ºC for 4 min). Cells were then resuspended in YEP-Galactose (peptone 2%, yeast extract 1%, galactose 1%) liquid media and incubated at 30 °C, with orbital agitation (200 rpm), for 6 h. For aSyn clearance experiments, $OD_{600\ nm}$ of the 6 h induced cultures was measured and the volume of yeast culture needed to inoculate a new culture with an initial standardized $OD_{600\ nm}$ = 0.2 (~$7x10^{6}$ cells/ml) was centrifuged (3000 rpm, at 30 ºC for 4 min). Cells were washed in PBS and resuspended in YEP-Glucose (peptone 2%, yeast extract 1%, glucose 2%) liquid media and incubated at 30 °C, with orbital agitation (200 rpm), for 6 h or 18 h.

For fluorescence microscopy or flow cytometry analysis, adenine was added to the growth media at a final concentration of 0.16 mg/mL to avoid background interactions by the red pigment production due to *ade2* auxotrophic marker of the used yeast strain. Adenine supplementation did not alter growth phenotypes of the tested yeast strains.

Flow cytometry

Yeast cell membrane integrity was evaluated with PI staining using a BD LSR Fortessa. Yeast cells were incubated with PI 5 µg/mL for 15 min. As a positive control, cells boiled for 10 min were used (data not shown).

Autophagy induction was determined measuring fluorescence intensity of mCherry-Atg8 under the regulation of the natural promoter [76] in cells co-expressing WT or S129A aSyn-GFP, using a BD FACSAria III equipped with a 561 nm laser for excitation and a 600 LP mirror in conjunction with a 610/20 BP filter for detection (BD Biosciences, San Jose, CA). Fluorescence intensity of WT or S129A aSyn-GFP was measured in simultaneous using a 488 nm laser for excitation and a 502 LP mirror in conjunction with a 530/30 BP filter for detection (BD Biosciences, San Jose, CA).

A minimum of 10.000 events were collected for each experiment. Data analysis was performed using FlowJo software (Tree Star Inc., Ahsland, OR, USA). Results were expressed as median fluorescence intensity (MFI).

Protein extraction and western blot analysis

For total protein extraction, yeast cells were lysed in Tris-HCl buffer pH 7.6 supplemented with protease and phosphatases inhibitor cocktail (Roche, Mannheim, Germany), with glass beads (3 cycles of 30 seconds in the beadbeater and 1 min on ice). Cell debris was removed with a smooth centrifugation (700 g, 3 min, 4 ºC) and the supernatant was collected. The supernatant was sonicated (10 seconds at 10 mA, Soniprep 150 from Sanyo). Protein concentrations were determined using the BCA protein assay kit (Thermo Fisher Scientific Inc, Illinois, USA). Protein sample buffer (200 mM Tris-HCl pH 6.8, 6% (w/v) 2-mercaptoethanol, 8% (w/v) SDS, 40% (v/v) glycerol, 0.4% (w/v) bromophenol blue) was added to each protein sample and heated for 10 min at 100 ºC before acrylamide gel loading. Protein samples were run in SDS-PAGE and were transferred to a nitrocellulose membrane using a Trans-Blot Turbo transfer system (Bio-Rad), as specified by the manufacturer. Immunoblotting was performed following standard procedures with the listed antibodies: aSyn (BD Transduction Laboratories, San Jose, CA, USA), pS129-aSyn (Wako Chemicals USA, Inc., Richmond VA, USA). PGK (Life Technologies, Grand Island, NY, USA) was used

as loading control.

The band intensity of the different immunoblots signals was estimated using ImageJ software (NIH, Bethesda, MD) and normalized against the corresponding PGK signal. In particular, aSyn levels were determined by calculating the ratio between aSyn/PGK and normalized to the control (mean ± SD); pS129-aSyn levels were determined by doing the ratio between both values: (pS129/PGK)/(aSyn/PGK) and normalized to the control (mean ± SD).

Size exclusion chromatography

Size exclusion-fast protein liquid chromatography (SEC-FPLC) was performed with total protein lysates from cells expressing WT or S129A aSyn-GFP extracted as described [77], centrifuged at 16,000g for 4 min and filter with 0.45 µM PVDF (Whatman) to remove any insoluble particles. Samples (3 mg of protein in final volume of 500 µL) were analyzed on a Superose 6 10/300 GL (GE Healthcare, Uppsala, Sweden) column using a FPLC system with UV-M II detector (GE Healthcare, Uppsala, Sweden). The samples were eluted with 50 mM ammonium acetate, pH 7.4 at a flow rate of 500 µL/min and the UV absorbance was monitored at 280 nm. To estimate the molecular weight of the protein samples, High Molecular Weight and Low Molecular Weight gel filtration calibration kits were used (GE Healthcare, Uppsala, Sweden). Fractions of 500 µL were collected, precipitated overnight at 4 °C in trichloroacetic acid, washed in acetone three times and resuspended in protein sample buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 10% (w/v) SDS, 0.1% (w/v) bromophenol blue), and were resolved by SDS-PAGE.

Fluorescence microscopy

The percentage of cells with aSyn inclusions, number of aSyn inclusions per cell and size of aSyn inclusions were determined by fluorescence microscopy using a Zeiss Axiovert 200M (Carl Zeiss) widefield fluorescence microscope equipped with a cooled CCD camera (Roper Scientific Coolsnap HQ) to acquire images containing at least 700 cells per strain, which were then manually counted using ImageJ. Yeast cells were grown as described above. At the indicated time points cells were collected by centrifugation and resuspended in PBS and 0.5% low melting agarose on a microscope slide.

Statistical Analysis

Statistical analyses were performed using one way ANOVA with Bonferroni's. P-value≤0.05 was considered statistically significant. Statistics were performed using SigmaStat (GraphPad Software Inc.).

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Supplementary material

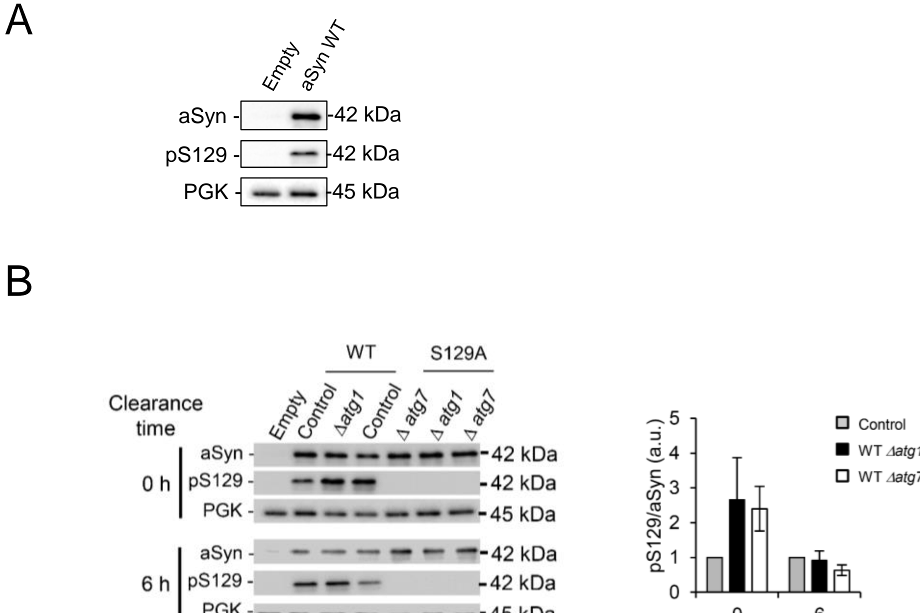

FIGURE S1. WT and S129A aSyn and pS129 levels. A) Protein levels of total aSyn and phosphorylation at the S129 residue (pS129) of the aSyn WT strain, after 6 h of aSyn induction in galactose medium. **B)** aSyn and pS129 levels determined in aSyn WT and S129A strain, at the indicated time points of aSyn clearance in control, Δ*atg1* and Δ*atg7* strains (left panel). The densitometry for pS129 levels are represented (right panel). Results shown are from one representative experiment from at least four independent experiments.

Chapter 2

(Poly)phenols protect from alpha-synuclein toxicity by promoting its clearance through autophagy

This chapter is based on the following manuscript:

(Poly)phenols protect from alpha-synuclein toxicity by reducing oxidative stress and promoting autophagy

Macedo D, Tavares L, McDougall DJ, Vicente Miranda H, Stewart D, Ferreira RB, Tenreiro S, Outeiro TF, Santos CN, *Human Molecular Genetics,* **2015** 24 (6): 1717-1732.

This chapter contains data in which the author of this dissertation executed the majority of the experiments. McDougall GJ performed the LC-MS and Tavares L analysed the resulting data.

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Abstract

Parkinson's disease (PD) is the most common movement neurodegenerative disorder and is associated with the aggregation of alpha-synuclein (aSyn) and oxidative stress, hallmarks of the disease. Although the precise molecular events underlying aSyn aggregation are still unclear, oxidative stress is known to contribute to this process. Therefore, agents that either prevent oxidative stress or inhibit aSyn toxicity are expected to constitute potential drug leads for PD. Both pre-clinical and clinical studies provided evidence that (poly)phenols, pure or in extracts, might protect against neurodegenerative disorders associated with oxidative stress in the brain.

In this study we analysed, for the first time, a (poly)phenol-enriched fraction (PEF) from leaves of *Corema album*, and used *in vitro* and cellular models to evaluate its effects on aSyn toxicity and aggregation. Interestingly, the PEF promoted the formation of non-toxic aSyn species *in vitro*, and inhibited aSyn toxicity and aggregation in cells, by promoting the autophagic flux and reducing oxidative stress. Thus, *C. album* (poly)phenols appear as promising cytoprotective compounds, modulating central events in the pathogenesis of PD, such as aSyn aggregation and the impairment of autophagy.

Ultimately, the understanding of the molecular effects of (poly)phenols will open novel opportunities for the exploitation of their beneficial effects and for drug development.

Introduction

Parkinson's disease (PD) is the second most common age-related neurodegenerative disease after Alzheimer's disease [1]. Despite intensive studies on the molecular basis of this disorder, we still lack a comprehensive understanding of the underlying mechanisms, compromising the development of effective therapeutic strategies. PD is deeply associated with the misfolding and aggregation of alpha-synuclein (aSyn), a presynaptic protein whose aggregation has also been linked to other disorders known as synucleinopathies [2, 3]. Thus, proteostasis dysfunction is central in PD and includes proteasomal, lysosomal and autophagic dysfunction. Although different pathways have been shown to contribute to the degradation of aSyn [4], autophagy is important in the clearance of aSyn aggregates [5, 6], and the proteasome is involved in the degradation of soluble and small oligomeric aSyn species [7, 8].

Currently, oligomeric aSyn species, rather than insoluble aggregated forms, are thought to be associated with aSyn cytotoxicity [9, 10]. The process of aSyn aggregation can be modulated by various internal and external stimuli, including oxidants and mitochondrial toxins [11], aSyn is highly susceptible to oxidative stress-induced posttranslational modifications that induce its misfolding and cytotoxicity [12, 13]. Moreover, oxidised aSyn is more prone to aggregation [14] and, on the other hand, aggregated aSyn induces oxidative stress [15], supporting a prominent role for oxidative stress in the onset and progression of PD. In addition, dopamine metabolism in the brain is also known to contribute to high levels of oxidative stress and neurodegeneration in PD [2, 3]. Therefore, the identification of novel agents that either prevent the production of reactive oxygen species (ROS) and/or inhibit aSyn aggregation are thought to be of particular interest.

(Poly)phenols, either as dietary (poly)phenols or nutraceutical supplements, are known for their protection against oxidative damage in the brain [16-18], suggesting they may be useful modifiers of aSyn aggregation [19]. These phytochemicals occur naturally in plants and epidemiological, pre-clinical and clinical studies showed their importance for human health as they reduce the incidence and prevalence of cardiovascular diseases, cancer, diabetes, inflammation and age-related disorders [16, 19, 20].

(Poly)phenols were initially thought to protect cells against oxidative damage through the scavenging of free radicals. However, several other mechanisms underlying the neuroprotective action of (poly)phenols have been recently described, such as iron chelation, regulation of calcium homeostasis, activation of signalling cascades, promotion of amyloid precursor protein (APP) processing and reduction of β-amyloid fibril formation [19, 21].

Several cellular models have been extensively used to study the molecular basis of different neurodegenerative disorders [22-24]. Here, we tested (poly)phenol-enriched fractions (PEFs) from leaves or fruits of *Arbutus unedo* and *Corema. album* against aSyn aggregation and toxicity in yeast and human cell models of PD [23, 25]. Leaves from *Ginkgo biloba*, and fruits from *Rubus idaeus* were also analysed, due to their known beneficial effects [26-29]. *A. unedo* is a native Mediterranean species with reported antioxidant and beneficial health properties [28, 30, 31], while the bioactivities from *C. album* native from Iberia Peninsula are poorly characterized, representing a pool of phytochemicals yet to be explored.

This study demonstrate that (poly)phenols from leaves and fruits of *A. unedo* and *C. album* protect yeast cells from general oxidative stress and also from aSyn-induced toxicity, two major hallmarks of PD. Interestingly, PEFs from *C. album* leaves, characterized for the first time in this study, were the most promising of the PEFs tested. These presented a higher protective capacity than *G. biloba*, a species recognized for its beneficial health properties [29]. Here we show that the mechanisms underlying *C. album* leaf PEF protection against aSyn-induced toxicity were associated with its ability to stabilize aSyn non-toxic oligomeric species *in vitro*, reduce ROS levels and increase the autophagic function, which is compromised by aSyn expression. These findings suggest that *C. album* leaf (poly)phenols are putative therapeutic agents for preventing aSyn toxicity in PD and other synucleinopathies.

Results

Chemical characterization of PEFs

PEFs, clean of organic acids, sugars and minerals, were obtained from fruits and leaves of *A. unedo* and *C. album*, leaves of *G. biloba*, and fruits of *R. idaeus* (the latest two used as a reference due to their well-established properties) [26-29]. PEFs were then assessed for total phenolic content and *in vitro* antioxidant capacity for peroxyl radical (Table 1), a reactive oxygen species with biological relevance in human physiology [32]. *C. album* leaf PEF presented the higher antioxidant activity, however *A. unedo* fruit PEF presented the highest antioxidant potential (antioxidant capacity per total phenol content) (Table 1).

The differences observed in the PEFs antioxidant capacity arise from its chemical composition. In order to identify the main compounds present in the plants under study, PEFs were analyzed by HPLC-PDA-ESI-MS (Fig. S1 and Table S1-S6).

Regarding the PEFs from leaves, the main components of *G. biloba* were flavonol glycosides, biflavonoids, terpenes and flavones, in agreement with previous studies [33]. *A. unedo* leaves contained mainly gallic acid derivatives [34], catechin, tannins and flavonol derivatives. *C. album* leaves presented flavonol derivatives, especially myricetin, (epi)catechin and proanthocyanidins. It also contained a range of late-eluting components with MS properties suggesting to be stilbene derivatives (Fig. S1).

A. unedo fruits yielded mainly gallic acid derivatives (also common in this species leaves) [34], conjugated with either quinic acid, shikimic acid, or glucose. Moreover anthocyanins, quercetin and ellagic acid derivatives were identified, as in [28, 34, 35]. *R. idaeus* fruits were dominated by anthocyanins and ellagitannins (Sanguiin H6 and Lambertianin C), with a number of other minor components [35, 36]. *C. album* fruits, still poorly characterized, were mainly composed of derivatives of chlorogenic acid and flavonols, in agreement with the literature [34, 37]. The main differences obtained between this fruit and the other two analyzed was the absence of anthocyanins, ellagic acid or ellagitannins and the presence of myricetin, kaempferol and caffeic acid derivatives.

Table 1. Total phenol content and *in vitro* peroxyl radical scavenging activity of PEFs.

	Species	Antioxidant capacity # (µmol TE.g^{-1} dw)	Total phenol content # (mg GAE.g^{-1} dw)	Antioxidant capacity per total phenol content #(µmol TE.mg^{-1} GAE)
Leaves	*G. biloba*	2162.94±207.32	88.83±14.00	24.35±1.50
	A. unedo	2331.37±212.35 nd	121.77±11.25**	19.15±0.02 ***
	C. album	2886.19±151.65 ***	112.35±3.96*	25.69±0.44 nd
Fruits	*R. idaeus*	444.57±19.25	13.64±0.09	32.60±1.20
	A. unedo	1498.36±52.46 ***	10.49±0.08 ***	142.78±3.90***
	C. album	956.19±54.46 **	15.81±0.12***	60.48±3.09***

#Values represent the mean ± SD of three independent experiments. The values of each matrix were statistically compared with the value of the respective comparison species (*G. biloba* for leaves and *R. idaeus* for fruits). Statistically significant differences are shown, *p<0.05, **p<0.01, ***p<0.001 or nd as not different for p<0.05.

(Poly)phenols reduce the formation of aSyn inclusions and its cytotoxicity in yeast

We used an established yeast model of aSyn aggregation and toxicity [25] to screen for PEFs ability to modulate aSyn toxicity. Yeast cells pre-treated with PEFs for 6 h, were further incubated with PEFs in glucose (control) or galactose medium, where aSyn expression is induced. We monitored cell growth (Fig. 1A), determined cell metabolic capacity (Fig. 1B) and quantified the percentage of cells displaying aSyn inclusions (Fig. 1C).

The concentrations tested were selected based on the analysis of the growth curves of BY4741 (WT, control) strain and the determination of the *lag* and doubling time of cells treated with various concentrations of PEFs (Fig. S2). *Δyap1*, *Δsod1* and AD1-8 strains were also studied for comparison, as these strains bear deletions in different genes related to oxidative stress tolerance and compound permeability [28, 35] (Fig. S2). At the lower concentration the PEFs did no alter the growth parameters of the WT yeast strain, not expressing aSyn. As expected, the expression of aSyn drastically decreased the growth of BY4741 cells (Fig. 1A). PEFs enhanced the growth of the yeast cells expressing aSyn, with

the exception of *A. unedo* leaf and *C. album* fruit PEFs (Fig. 1A). After a longer *lag* phase, cells exposed to *R. idaeus* fruit, *G. biloba* leaf, *A. unedo* fruit and *C. album* leaf recovered and achieved growth rates similar to those of the control cells (Fig. 1A). The metabolic capacity assay, based on the ability of living cells to convert resazurin to resorufin, showed that *C. album* leaf PEF promoted the highest level of protection against aSyn toxicity, followed by *C. album* fruit, *A. unedo* fruit and *G. biloba* leaf (Fig. 1B).

The formation of inclusions is related with aSyn-induced toxicity with concomitant reduction of cellular viability [25, 38]. To assess if the enhancement in cell viability was followed by alterations in the sub-cellular localization of aSyn, cells expressing aSyn fused with GFP (aSyn-GFP) were treated with PEFs and the percentage of cells with aSyn inclusions was monitored by fluorescence microscopy (Fig. 1C). Interestingly, treatment with *C. album* leaf PEF reduced the percentage of cells displaying aSyn inclusions. *A. unedo* leaves also induced a small decrease in aSyn inclusion formation (Fig. 1C).

The effect of the PEFs on oxidative stress was also analysed by subjecting BY4741 WT, *Δyap1*, *Δsod1* and AD1-8 cells to H_2O_2 (Fig. S3). *C. album* leaf PEF induced a mild protection in *sod1* and AD1-8 cells.

Overall, these results suggest that *C. album* leaves PEF is the strongest cytoprotective agent in the yeast model of PD. Interestingly, the PEFs presented different composition and protective capacities, affecting distinct parameters. The PEF from *C. album* leaves counteracted the cytotoxicity induced by aSyn expression (Fig. 1A and B), was capable of reducing the percentage of cells displaying aSyn inclusions (Fig. 1C), and enhanced the growth phenotype of yeast strain under H_2O_2-induced toxicity (Fig. S3).

C. album leaf PEF comprises a complex mixture of (poly)phenols (Fig. S1 and Table S3). Myricetin-3-*O*-galactoside (peak 4, Fig. S1 and Table S3), myricetin-3-*O*-glucoside (peak 5, Fig. S1 and Table S3) and quercetin-3-*O*-galactoside (peak 13, Fig. S1 and Table S3) are three of the main identified (poly)phenols. In order to verify if these compounds could be responsible for the observed protection against aSyn toxicity, we compared their activity with *C. album* leaf PEF in the yeast PD model (Fig. 1D). Yeast cells expressing aSyn were treated with a range of concentration from 0.5 to 30 $\mu g.\ mL^{-1}$ of the isolated compounds dissolved in the same medium as PEF, and viability was assessed by flow cytometry (FCM)

using propidium iodide (PI), a fluorescent molecule that is only absorbed by cells that have lost membrane integrity. At the range of concentrations tested the pure compounds separately did not show any efficacy in reducing cell death (in comparison to non-treated cells expressing aSyn). Remarkably, *C. album* leaf PEF caused a dose-dependent reduction of aSyn toxicity (≈ 40%).

Protection against aSyn cytotoxicity is mediated by the reduction of ROS

To further characterize the effect of *C. album* leaf PEF on aSyn-induced toxicity and aggregation, yeast cells expressing aSyn-GFP were treated with this PEF and analyzed by FCM for aSyn-GFP and PI fluorescence (Fig. 2A). Cell doublets were excluded from all flow cytometry analyses. aSyn expressing cells presented a higher percentage of PI positive cells compared to the control where aSyn was not expressed (Fig. 2A and B). This is in agreement with the previous results (Fig. 1A and B) and with the known cytotoxic effects of the human protein [11, 25].

When cells expressing aSyn were treated with *C. album* leaf PEF, an increase in cell viability was observed (Fig. 2A and B). Similarly, this PEF ameliorated the growth phenotype of these cells (Fig. 2C). These observations are in agreement with the detected increase in metabolic capacity of the cells (Fig. 1B).

Using FCM we observed a reduction in aSyn median fluorescence intensity (MFI) mediated by *C. album* leaf PEF (Fig. 1D). This was correlated with the ability of this PEF to reduce the percentage of cells with aSyn inclusions by 28.2%, as confirmed by fluorescence microscopy (Fig. 1C and 2E respectively).

Interestingly, cells expressing aSyn-GFP presented two fluorescence intensity peaks corresponding to two different sub-populations (regions marked as 1 and 2 in Fig. 2D). The higher MFI observed for sub-population 2 is due to the presence of aSyn inclusions with higher fluorescence intensity. In fact, GFP fluorescence intensity was stronger in cells presenting aSyn inclusions (Fig. 2E, cells marked with 2) than in cells where aSyn was localized at the cell membrane (Fig. 2E, cells marked with 1). These different intensities were clearly visible when cells were observed by fluorescence microscopy (Fig. 2E) [38]. *C. album* PEF reduced the population with aSyn inclusions (sub-population 2) by 25.2% (Fig. 2D).

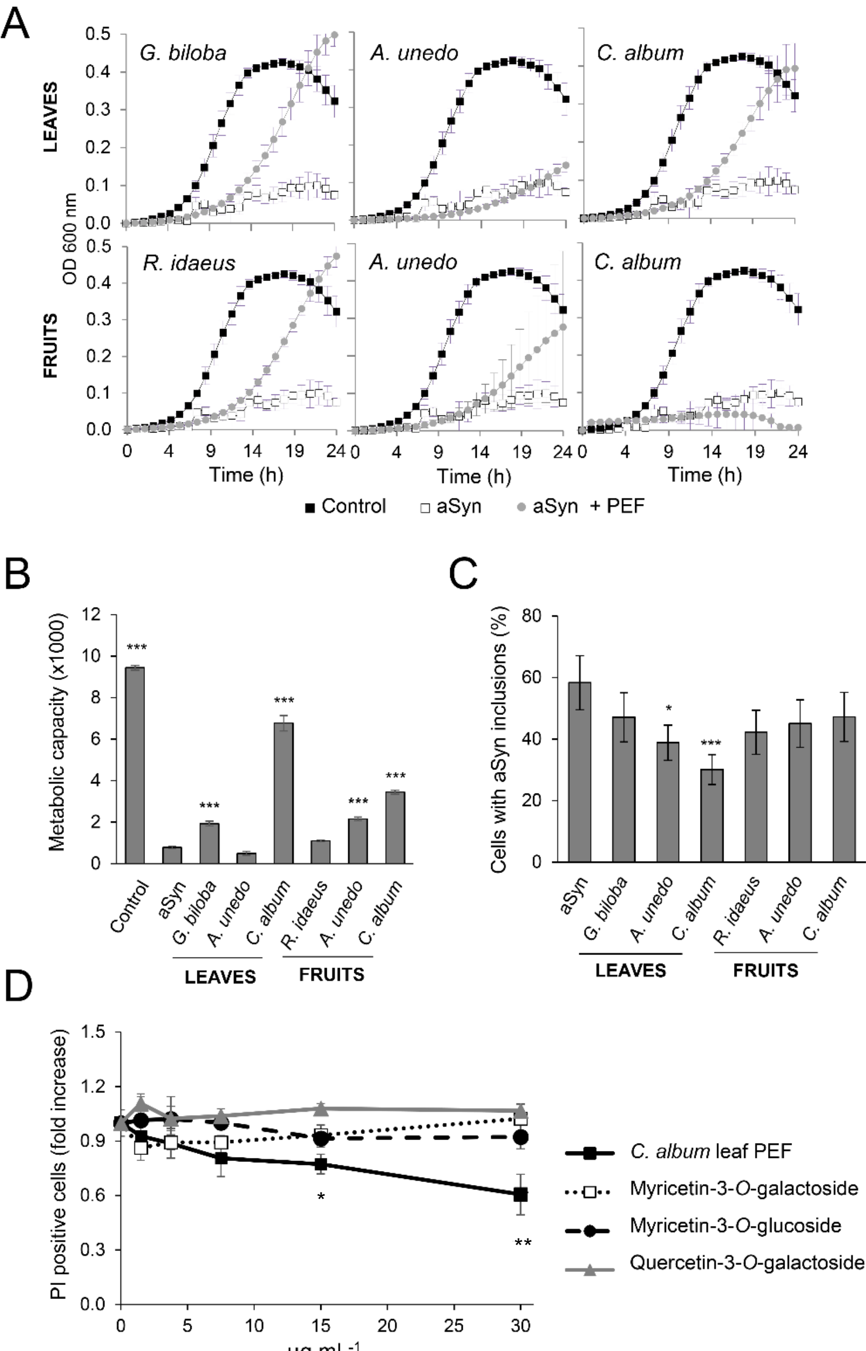

A
LEAVES
FRUITS
OD 600 nm
G. biloba
A. unedo
C. album
R. idaeus
A. unedo
C. album
Time (h)
Control
aSyn
aSyn + PEF
B
Metabolic capacity (x1000)
Control
aSyn
G. biloba
A. unedo
C. album
R. idaeus
A. unedo
C. album
LEAVES
FRUITS
C
Cells with aSyn inclusions (%)
aSyn
G. biloba
A. unedo
C. album
R. idaeus
A. unedo
C. album
LEAVES
FRUITS
D
PI positive cells (fold increase)
μg.mL-1
C. album leaf PEF
Myricetin-3-O-galactoside
Myricetin-3-O-glucoside
Quercetin-3-O-galactoside

FIGURE 1. PEFs protect yeast from aSyn-induced toxicity and reduce the percentage of cells with aSyn inclusions. BY4741 cells transformed with the aSyn encoding plasmid were treated with the indicated PEFs for 6 h in raffinose medium, prior to treatment with PEFs in glucose (Control, aSyn OFF) or galactose medium (aSyn ON). **A)** Growth curves of cells subjected to the described treatment for 24 h: Control (■), aSyn (□) or aSyn with PEFs (●). **B)** Cells metabolic capacity, expressed in arbitrary units (x1000), assessed at 6 h after aSyn expression induction. **C)** Percentage of cells with aSyn inclusions, determined by fluorescence microscopy imaging, after 6 h of aSyn-GFP expression induction. **D) V**iability of cells subjected to the treatment with *C. album* leaf PEF (■) or the pure compounds myricetin-3-*O*-galactoside (□), myricetin-3-*O*-glucoside (●) or quercetin-3-*O*-galactoside (▲), at the indicated concentrations. Viability was evaluated by percentage of PI positive cells normalized to aSyn untreated cells (fold increase), assessed by flow cytometry. A representative results is shown and values represent the mean ± SD of at least three independent experiments. Statistically significant different results from cells expressing aSyn not submitted to PEFs treatment are shown, *p<0.05, **p<0.01 or ***p<0.001.

Together with the reduction of aSyn inclusions (Fig. 1C) and aSyn fluorescence intensity (Fig. 2D and E), these data confirm that *C. album* leaf polyphenols restore the sub-cellular localization of aSyn to the membrane. Next, aSyn expression was analyzed by immunoblot (Fig. 2F). Importantly, we found that these (poly)phenols did not alter the levels of aSyn.

Oxidative stress is a known trigger of aSyn misfolding [12, 13]. ROS levels were determined by FCM using 2',7'-dichlorfluorescein-diacetate (DCFHDA) (Fig. 2G), an indicator of the general oxidative state of the cell [39]. aSyn expression in yeast induced an increase in ROS accumulation (Fig. 2G) as previously described [40]. PEF treatment reduced the basal levels of ROS both in the control and in cells expressing aSyn.

To gain insight into the redox homeostasis status, superoxide radical levels were also evaluated as an indicator of mitochondrial metabolic function [41], since these organelles are damaged by aSyn-induced toxicity [42]. Superoxide was analyzed by FCM using a specific probe, dihydroethidium (DHE) [39], and the results indicated that aSyn expression led to an increase in superoxide production (Fig. 2H). Treatment with PEF reduces the basal levels of this radical in control cells as well as in cells expressing aSyn (Fig. 2H). Thus, the protective role of *C. album* PEF may be related with the improvement of redox homeostasis and mitochondrial function.

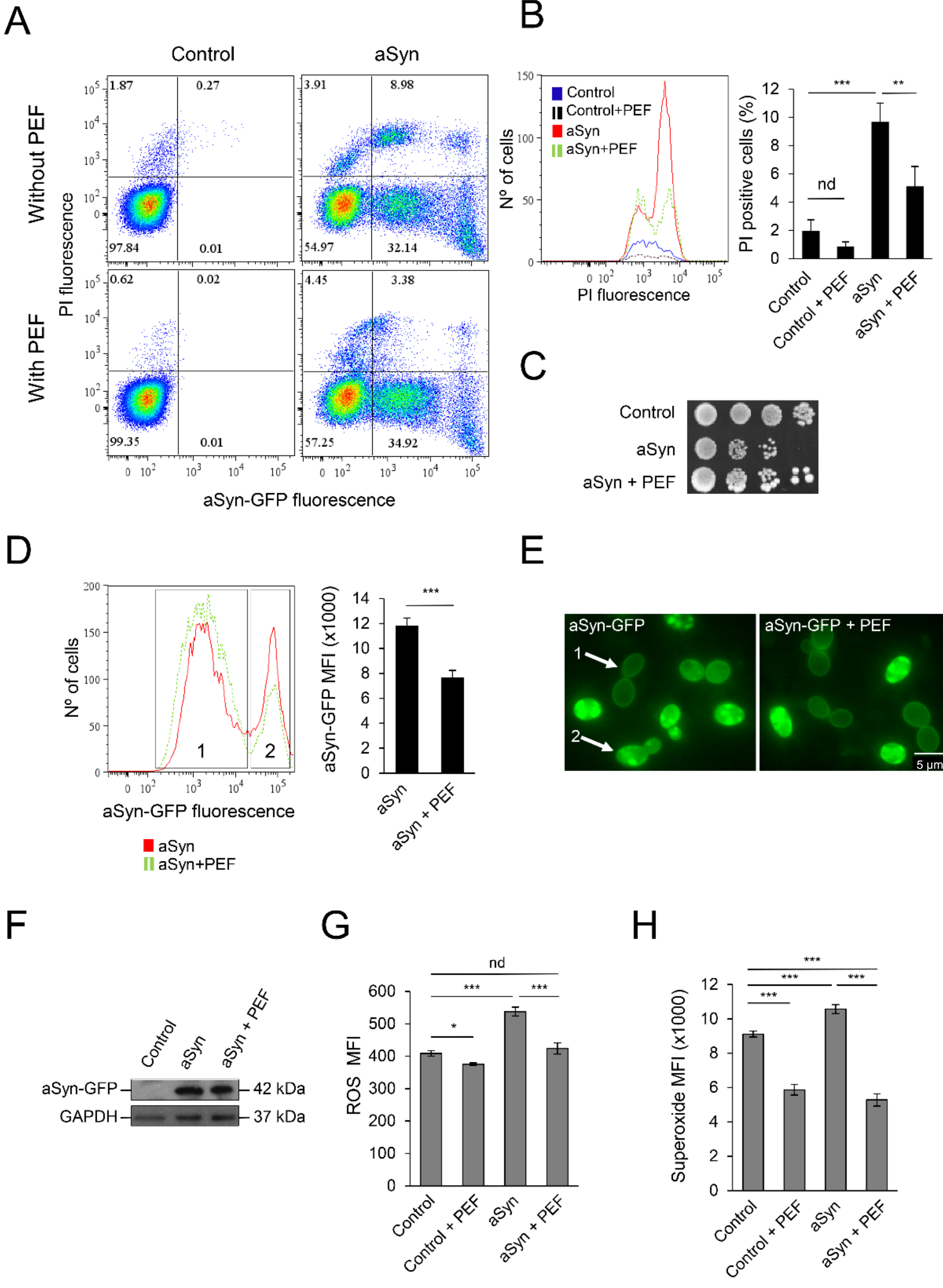

A
Control
aSyn
Without PEF
With PEF
PI fluorescence
aSyn-GFP fluorescence
1.87
0.27
97.84
0.01
3.91
8.98
54.97
32.14
0.62
0.02
99.35
0.01
4.45
3.38
57.25
34.92
B
Nº of cells
PI fluorescence
Control
Control+PEF
aSyn
aSyn+PEF
PI positive cells (%)
nd
***
**
Control
Control + PEF
aSyn
aSyn + PEF
C
Control
aSyn
aSyn + PEF
D
Nº of cells
aSyn-GFP fluorescence
aSyn
aSyn+PEF
aSyn-GFP MFI (x1000)
***
aSyn
aSyn + PEF
E
aSyn-GFP
aSyn-GFP + PEF
5 µm
F
Control
aSyn
aSyn + PEF
aSyn-GFP
GAPDH
42 kDa
37 kDa
G
ROS MFI
nd
***
***
*
Control
Control + PEF
aSyn
aSyn + PEF
H
Superoxide MFI (x1000)
***
Control
Control + PEF
aSyn
aSyn + PEF

FIGURE 2. *C. album* leaf PEF reduces aSyn-induced toxicity, aggregation and ROS levels. BY4741 cells were treated with *C. album* leaf PEF for 6 h in raffinose medium, prior to treatment for 12 h with PEF in galactose medium (aSyn ON). Cells transformed with the aSyn empty vector were use as control. **A)** Cell viability and aSyn expression evaluated by PI *versus* aSyn-GFP fluorescence, respectively, assessed by flow cytometry (FCM). **B)** Histograms of number of cells *versus* PI fluorescence intensity assessed by FCM, showing only the PI positive population (left panel), and the correspondent percentage of PI positive cells (right panel). **C)** Cell viability assessed by spot assays of cells subjected to the treatment. **D)** Histogram of the number of cells *versus* aSyn-GFP median fluorescence intensity (MFI), showing only the GFP positive population (left panel) and the correspondent MFI values (right panel), assessed by FCM. Regions marked represent sub-population 1 and sub-population 2 of cells exhibiting different aSyn-GFP fluorescence intensity. **E)** Fluorescence microscopy images of cells subjected to the treatment, with aSyn localized either in the membrane or in intracellular inclusions (cells marked with 1 and 2, respectively). **F)** aSyn expression levels of cells subjected to the treatment (GAPDH was used as loading control), assessed by western blot. **G)** Reactive oxygen species (ROS) MFI, assessed by FCM using the DCFHDA probe. **H)** Superoxide radical MFI assessed by FCM using the DHE probe. A representative results is shown and values represent the mean ± SD of at least three independent experiments. Statistically significant differences between the indicated treatments are shown, *$p<0.05$, **$p<0.01$, ***$p<0.001$ or nd as not different for $p<0.05$.

(Poly)phenols induce the clearance of aSyn by promoting autophagy

One of the mechanisms implicated in the pathogenesis of PD is the impairment of the protein clearance pathways [4, 43]. In yeast, the clearance of aSyn inclusions has been associated with both ubiquitin-proteasome system (UPS) and autophagy [44]. First, we evaluated aSyn clearance at 24 h by western blotting (Fig. 3A). Interestingly, cells treated with PEF presented lower levels of aSyn at 24 h of clearance, suggesting that the (poly)phenols promoted the degradation of aSyn. Thus, we next asked whether *C. album* leaf PEF protected against aSyn toxicity by interfering with the activity of aSyn proteostasis pathways. The effect of PEF on the proteasome activity was analyzed using an unstable GFP reporter (GFPu) consisting of a fusion of GFP with a constitutive degradation signal (CL-1) that promotes its rapid degradation by the UPS [25]. FCM analysis was performed in cells co-expressing aSyn and GFPu and we found that upon aSyn expression the levels of GFPu levels increased, as previously reported (Fig. 3B) [25]. Treatment with *C. album* leaf PEF did not affect GFPu levels, independently of aSyn expression (Fig. 3B).

Subsequently, we tested whether autophagy was affected using GFP-Atg8 as a reporter [45, 46]. Atg8 is one of the key molecules involved in autophagy. Its conjugation to the autophagosomal membrane, through an ubiquitin-like conjugation system, is essential for autophagy initiation [46].

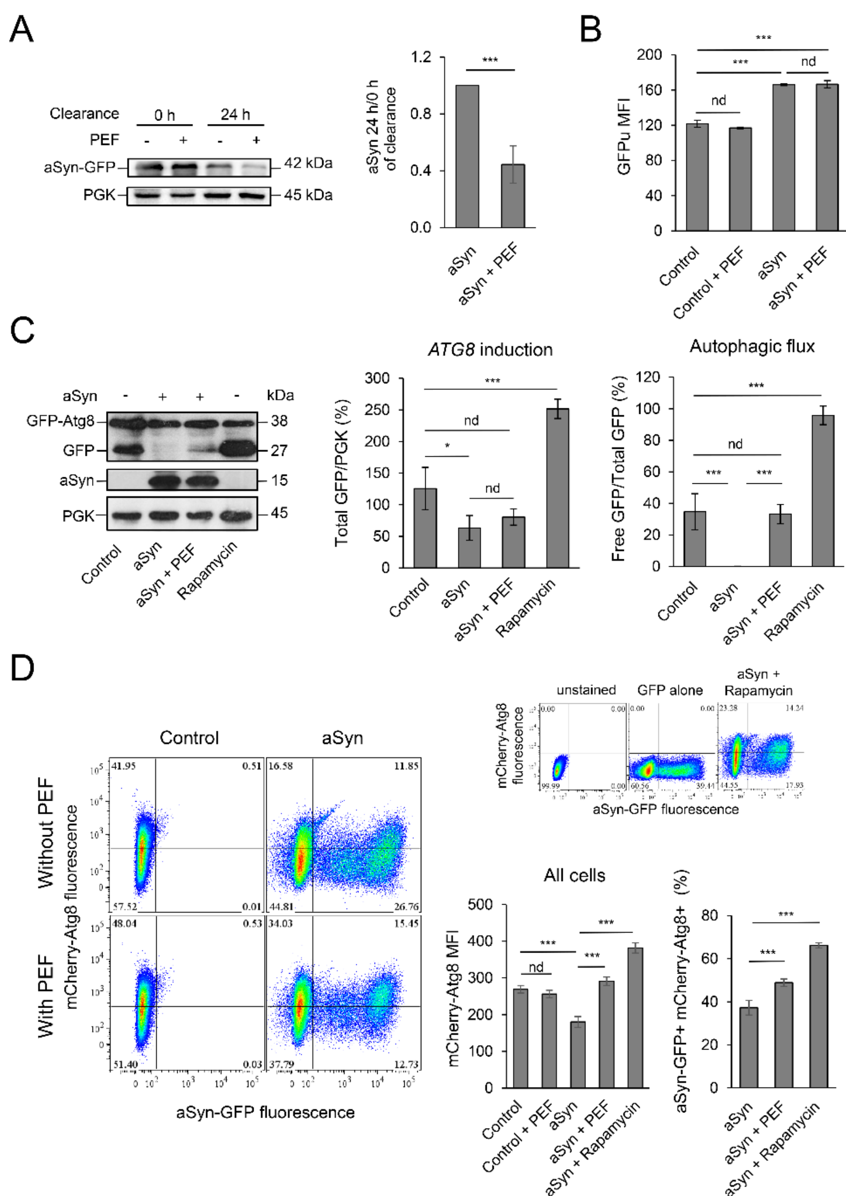


FIGURE 3. *C. album* leaf PEF promotes the clearance of aSyn by stimulating autophagy without changing the proteasomal function. BY4741 cells were treated with *C. album* leaf PEF for 6 h in raffinose medium, prior to treatment for 12 h with PEF in galactose medium (aSyn ON). Cells transformed with the aSyn empty vector were use as control. **A)** Clearance of aSyn evaluated by western blotting at the indicated time points (left panel) and respective densitometry normalized to 0 h of clearance (right panel).PGK was used for normalization. **B)** Proteasome impairment evaluated

by flow cytometry (FCM) using GFPu median fluorescence intensity (MFI). **C)** Autophagy evaluated by GFP-Atg8 processing assay assessed by western blot (left panel). *ATG8* induction quantified by the total GFP signal (GFP-Atg8 and free GFP signal, detected with anti-GFP) (middle panel); autophagic flux quantified by measuring the vacuolar degradation of the Atg8 domain reporter (ratio of free GFP to total GFP signal) (right panel). Rapamycin was used as a positive control. **D)** Autophagy and aSyn expression evaluated by mCherry-Atg8 *versus* aSyn-GFP fluorescence, respectively, assessed by FCM (left panel); corresponding mCherry-Atg8 MFI (middle panel); and percentage of cells aSyn-GFP and mCherry-Atg8 positive, determined only in the aSyn-GFP positive population of cells (right panel). A representative results is shown and values represent the mean ± SD of at least three independent experiments. In the upper panel the staining controls are represented. Statistically significant differences between the indicated treatments are shown, *p<0.05, **p<0.01, ***p<0.001 or nd as not different for p<0.05.

The GFP-Atg8 processing assay showed that aSyn expression lead to a significant reduction on both *ATG8* induction and autophagic flux (Fig. 3C), suggesting an interference in autophagy function. Interestingly, when aSyn expressing cells were treated with *C. album* leaf PEF, the autophagic flux was restored to control levels (Fig. 3C). Although we observe a tendency to increased autophagic induction and flux in control cells exposed to PEF in the tested conditions, this effect is not statistical significant (Fig. S4). Autophagy was also monitored by flow cytometry (FCM) using mCherry-Atg8*,* as mCherry fluorescence is more stable than GFP at vacuolar pH [46] (Fig. 3D). We observed a reduction in mCherry-Atg8 MFI upon aSyn expression, and an increase in cells treated with rapamycin (Fig. 3D, middle panel), in agreement with the GFP-Atg8 processing assay (Fig. 3C).

Interestingly, with GFP-Atg8 processing assay we also detected an increase in mCherry-Atg8 MFI in cells expressing aSyn treated with PEF (Fig. 3D, middle panel), and an increase in the percentage of cells aSyn-GFP and mCherry-Atg8 positive (Fig. 3D, right panel). By analyzing aSyn expressing cells using FCM, we observed difference in *ATG8* induction that was not detectable in the GFP-Atg8 processing assay (Fig. 3C), mainly due to the possibility of analyzing only the population of cells expressing aSyn. These observations indicate that (poly)phenols stimulate autophagy in yeast cells expressing aSyn (Fig. 3C and D).

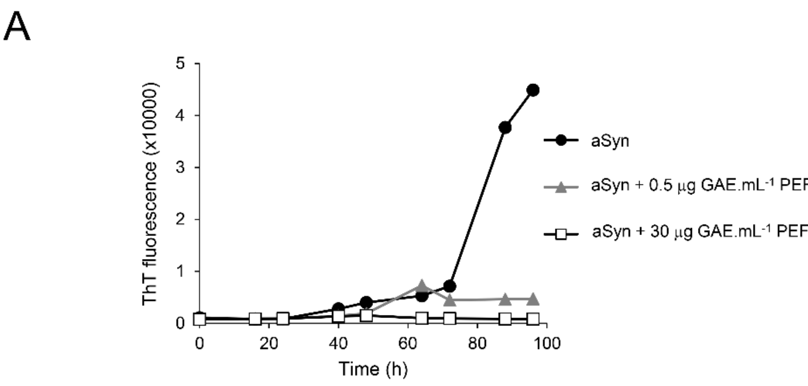
(Poly)phenols inhibit aSyn fibrillization in vitro and promote the formation of stable, non-toxic oligomeric species

In order to assess if the protection observed was due to a direct modulation of aSyn aggregation, we investigated the fibrillization of recombinant aSyn in the presence of 0.5 or 30 µg GAE. mL^{-1} of *C. album* leaf PEF (Fig. 4A). The kinetics of aSyn fibril formation was monitored using thioflavin T (ThT) as previously described [47]. Upon aSyn fibrillization, we observed the expected increase in ThT fluorescence signal, confirming the formation of amyloid-like fibrils. When recombinant aSyn was incubated with *C. album* leaf PEF, no significant increase in ThT fluorescence was observed (more evident in the presence of 30 ug GAE. mL^{-1}), suggesting that fibril formation was inhibited.

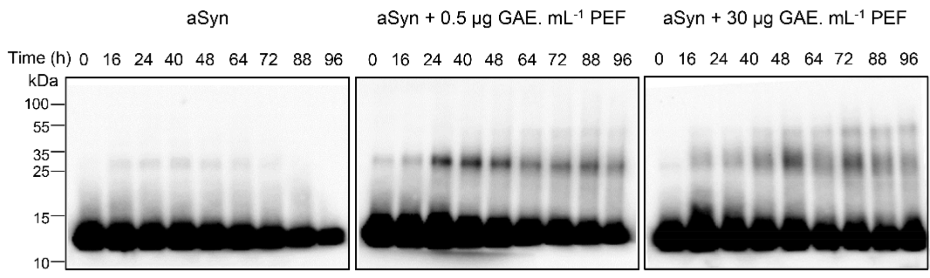
The biochemical nature of the aSyn species was further evaluated by SDS-PAGE (Fig 4B). We observed that *C. album* leaf PEF promoted the formation of SDS-stable, high-molecular oligomeric aSyn species, an effect that was previously described with pure (poly)phenols [48, 49].

To evaluate the toxicity of these oligomeric aSyn species obtained after *C. album* leaf PEF incubation, human neuroglioma H4 cells were treated with 1 µM of aSyn species and LDH activity was assessed in the medium as a measure of membrane integrity (Fig. 4C). We compared the toxicity of aSyn species before (0 h) and after (48 h) fibrillization in the presence/absence of PEF. Cells treated with the vehicle or PEF alone were used as controls. While the aSyn species formed in the absence of PEF were toxic, those formed in the presence of *C. album* leaf PEF were not (Fig. 4C). In agreement, a previous report showed that stable oligomeric aSyn species formed in the presence of EGCG were not toxic [48]. To gain further insight into the effect of PEF on aSyn, we analysed the Triton X-100 detergent solubility of the aSyn species formed in yeast cells (Fig. 4D). The treatment with PEF resulted in a reduction of aSyn in the soluble fraction and consequent increase in the insoluble fraction. These results are consistent with the *in vitro* ThT assay, suggesting that (poly)phenols promoted the stabilization of oligomeric species both *in vitro* and in yeast cells.

A



B



C D

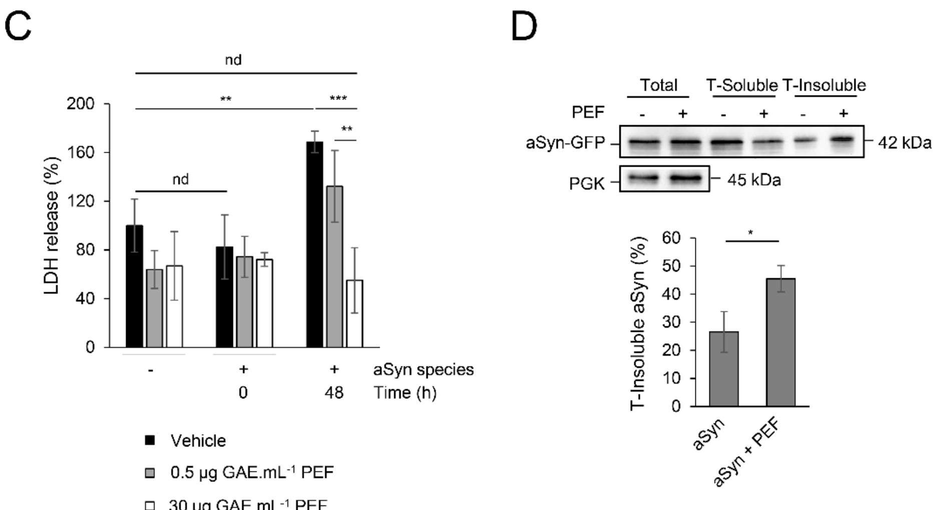
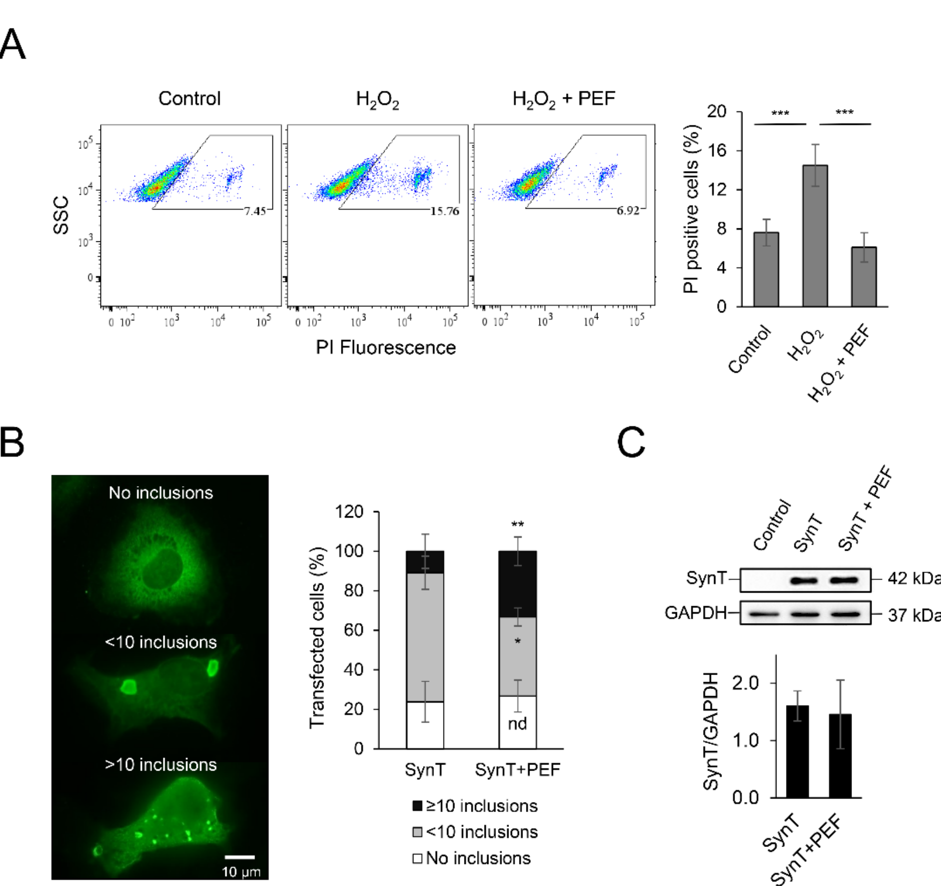


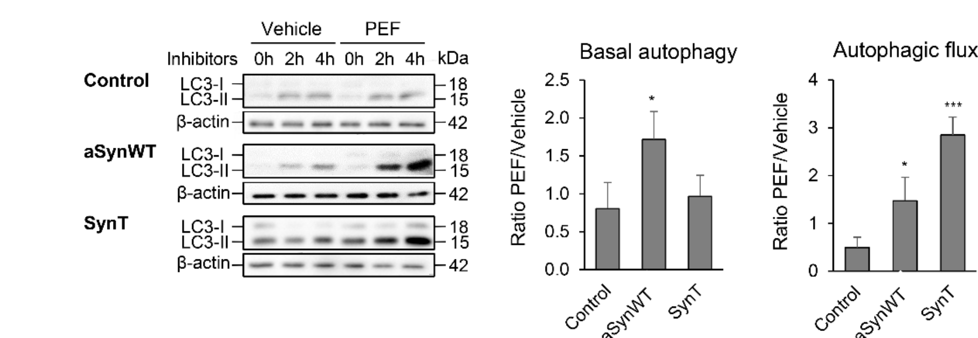
FIGURE 4. *C. album* leaf PEF reduces aSyn fibrillization and promotes the formation of non-toxic aSyn species. aSyn (70 μM) was allowed to fibrillization in the presence of *C. album* leaf PEF, and samples were collected over time. **A)** Fibril formation was monitored by the increase in thioflavin T (ThT) fluorescence. aSyn alone (●), with 0.5 (▲) or 30 (□) μg GAE.mL^{-1} *C. album* leaf PEF. **B)** Western blot of aSyn species separated by SDS-PAGE. **C)** Effect of aSyn species in H4 viability, assessed by LDH release. H4 cells were treated for 6 h with aSyn resulting species from vehicle, aSyn 0.5 or 30 μg GAE.mL^{-1} *C. album* leaf PEF treatment obtained before (0h) and after (48h) fibrillization. Cells treated with vehicle or PEF alone were used as controls. Results are normalized to the vehicle control cells. **D)** Triton-X 100 soluble (T-Soluble) and Triton-X 100 insoluble (T-Insoluble) fractions of yeast cells expressing aSyn treated with PEF, assessed by western blotting (upper panel) and determination by densitometry analysis of the percentage of T-Insoluble aSyn (lower panel). Values are normalized to the total fraction. A representative result is shown and values represent the mean ± SD of at least three independent experiments. Statistically significant differences between the indicated treatments are shown, *$p<0.05$, **$p<0.01$, ***$p<0.001$ or nd as not different for $p<0.05$.

(Poly)phenols protect human cells from H_2O_2 induced toxicity, modulate aSyn aggregation and promote autophagy

The protective capacity of *C. album* leaf PEF was studied in human H4 cells treated with H_2O_2 (Fig. 5A). The pre-treatment with PEF protected cells from oxidative stress-induced cell death, as observed by the reduction of PI positive cells (Fig. 5A). These data show that the PEF protects human cells from oxidative stress, in line with the observed reduction of ROS and superoxide radical in yeast (Fig. 2G and H). When studying aSyn aggregation we observed that the number of aSyn inclusions per cell was modulated by the PEF (Fig 5B). Specifically, PEF-treatment increases the percentage of cells presenting more than 10 inclusions. Importantly, the effect was independent of aSyn levels (Fig. 5C).

Subsequently, we evaluated autophagy by measuring LC3-II levels in human H4 cells (Fig. 5D, left panel), LC3-II is the mammalian homologue of ATG8 and is used as an autophagosomal marker [46, 50]. In cells expressing aSynWT treated with PEF we observed a significant increase in the LC3-II basal levels (Fig 5D, middle panel). Furthermore, the autophagic flux was evaluated by determining the accumulation of LC3-II upon impairment of autophagy with lysosomal inhibitors [46, 50]. PEF treatment increased the autophagic flux in both aSynWT and SynT cells (right panel). In agreement with the observations in yeast these results also suggest that (poly)phenols promote autophagy in aSyn expressing cells (Fig. 3).





Chapter 2

FIGURE 5. *C. album* leaf PEF protects against H_2O_2-induced toxicity, modifies the distribution of aSyn inclusions and induces autophagy in H4 cells. A) H4 cells were pre-treated with *C. album* leaf PEF for 16 h, then subjected to 600 µM of H_2O_2 for 6 h. Viability was assessed by PI fluorescence *versus* side scatter (SSC) (left panel); correspondent percentage of PI positive cells (right panel), determined by flow cytometry. **B)** H4 cells were transfected with SynT and Synphilin-1-V5 (SynT) for 24 h and treated with PEF for 6 h, subsequently the percentage of cells with inclusions was determined by fluorescence microscopy (left panel). Cells were classified by cells without inclusions (□), with less than 10 inclusions (■), or with 10 or more inclusions (■) (right panel). **C)** aSyn levels of H4 cells transfected with empty vector (Control), co-transfected with SynT and Synphilin-1-V5 (SynT) or treated with 30 µg GAE.mL^{-1} *C. album* leaf PEF (SynT+PEF) cells, assessed by western blot (upper panel). GAPDH was used as loading control. Corresponding densitometry is presented (lower panel). **D)** Autophagy evaluated by LC3-II levels in PEF treated cells. Control cells (not expressing aSyn), cells expressing SynT and Synphilin-1-V5 (SynT) or cells expressing aSyn WT were treated with 20 mM NH_4Cl and 200 µM leupeptin (lysosomes inhibitors) for 2 h and 4 h. LC3 levels were determined by western blot (β-actin was used as loading control) (left panel); The basal levels of autophagy were measured (middle panel) and the autophagic flux was given by the difference between LC3-II levels in cells treated or not with lysosomes inhibitors for 4 h (right panel). Results are presented as the ratio of PEF treated and vehicle treated cells. A representative result is shown and values represent the mean ± SD of at least three independent experiments. Statistically significant differences between the indicated treatments are shown, *$p<0.05$, **$p<0.01$, ***$p<0.001$ or nd as not different for $p<0.05$.

Discussion

PD and other synucleinopathies are currently incurable disorders affecting a growing number of individuals, due to the aging of the human population. Thus, there is an urgent demand for the identification of novel strategies for therapeutic intervention. Oxidative stress has long been implicated in PD and other neurodegenerative diseases, constituting an attractive pathway for intervention. Given that public health policies are currently focused on the prevention of cognitive decline, through the promotion of physical exercise, cognitive and social activity, and natural nutrition and supplementation, (poly)phenols play an attractive role as they can easily be incorporated as dietary supplements [16, 17].

In this study PEFs from *A. unedo*, *C. album, G. biloba* and *R. idaeus* obtained from leaves and/or fruits were analysed by LC-MS and evaluated for *in vitro* antioxidant capacity. Furthermore, the cytoprotective activities of these PEFs were analysed using established yeast and human cell models of PD/synucleinopathies [22, 25].

Phenolic compounds, pure or in extracts, are of great interest in nutrition and medicine because of their beneficial effects on human health, demonstrated in both pre-clinical and clinical trials [16, 19, 29-31]. However, a detailed understanding of the chemical origin underlying the bioactivities of these plant species is essential for the rational use of these compounds. Berries are known for their high content in (poly)phenols and in particular anthocyanins, usually associated to their high antioxidant properties [51]. Interestingly, the berries analyzed in this study have distinct levels of this phenolic class that do not reflect on their *in vitro* antioxidant activity. In particular, PEFs from *A. unedo* and *C. album* fruits, with lower anthocyanin content, exhibited higher antioxidant capacity than *R. idaeus*, a species rich in anthocyanins. We identified other phenols, like gallic acid, chlorogenic acid derivatives and flavonols as contributors to the antioxidant capacity observed for that species

With respect to the leaves, *C. album* PEF, characterized here for the first time, presented the highest antioxidant capacity. The main difference between this PEF and the ones from *A. unedo* and *G. biloba* leaves is the content in myricetin glucosides and

stilbene derivatives. Stilbenes have emerged as promising molecules in human health, being resveratrol the most studied one [52].

S. cerevisiae was used as an eukaryotic cell model to study the bioactivity of PEFs. By using strains with distinct genetic backgrounds that resulted in different susceptibilities to oxidative stress and compound permeability [28, 35], we were able to conclude that a higher *in vitro* antioxidant capacity was not directly related to an effective *in vivo* antioxidant protection of the studied PEFs. This observation reinforces the idea that other mechanisms, in addition to radical scavenging activity, may be involved in the protection by (poly)phenols.

While the majority of PEFs counter balanced the absence of *SOD1*, only the leaf and fruit (poly)phenols from *A. unedo* were able to protect yeast cells upon *YAP1* deletion. This gene encodes a transcription factor that plays an important role in the oxidative stress response [53, 54]. These findings suggest a specific interaction between *A. unedo* (poly)phenols and the genes/enzymes regulated by Yap1p should be occurring, in order to allow cells to overcome its absence. Importantly, some flavonoids modulate the expression of known targets of Yap1p, namely γ-glutamylcysteine synthetase (Gsh1p) [55].

The diverse chemical composition of the PEFs and the distinct genetic background of the yeast strains tested might account for their different bioactivities. In fact, the cellular absorption of (poly)phenols may differ greatly and is influenced by factors such as chemical structure, which affects their biological properties, antioxidant activity, specific interactions with cell receptors and enzymes [56]. For instance, aglycone forms of (poly)phenols are more hydrophobic than its glycosylated forms which are highly hydrophilic. Previous studies showed that aglycone forms may have the ability to cross cellular membranes through diffusion, while the absorption of the (poly)phenols glucosides is thought to occur via transporters in mammalian cells [56-58]. In the PEFs tested in this study, the main compounds are glycosylated, except in *C. album* fruit that is characterized by the presence of non-glycosylated phenolic acids although conjugated to quinic acid [34].

To further characterize the bioactivities of the PEFs, we tested them in both yeast and human cell models of PD [23, 25]. The cytotoxicity induced by aSyn expression was evaluated by complementary approaches, with respect to cell viability, aSyn inclusion formation and ROS production.

Our data demonstrate that *C. album* leaf PEF was the most promising protective agent against both oxidative stress conditions and aSyn-induced toxicity. To investigate the mechanism of action of *C. album* leaf PEF we explored its effect on different cellular pathways related to aSyn toxicity (summarized in Fig. 6).

C. album leaf PEF enhances the viability of yeast cells while reducing the percentage of cells displaying aSyn inclusions and promoting its clearance. In human H4 cells, the same PEF protects from H_2O_2-induced toxicity and modulates the distribution of aSyn inclusions, increasing the percentage of cells with more than 10 inclusions. Our studies using recombinant aSyn revealed than *C. album* leaf PEF directly interferes with fibrillization and promotes the formation of stable high-molecular weight species. Interestingly, these species are less toxic when added exogenously to cells. The *in vitro* fibrillization observations goes in line with the increased insolubility of aSyn species promoted by PEF, in the yeast PD model. Thus, by stabilizing these non-toxic species, the PEF may be reducing the formation of more toxic species [10, 49]. In fact, previous studies investigated the direct effect of isolated (poly)phenols on amyloid fibril formation [21, 47, 59]. Myricetin and quercetin were described to efficiently inhibit aSyn fibrillization [10]. In *C. album* leafs, the main compounds identified were myricetin-3-*O*-glucoside, myricetin-3-*O*-galactoside and quercetin-3-*O*-glucoside. We compared these isolated compounds with *C. album* leaf PEF for their capacity to protect against aSyn-induced toxicity. The isolated compounds did not reproduce the PEF effect, suggesting that synergistic effects between PEF compounds are necessary and/or that other less-abundant compounds contribute to the overall protective effect. In fact, in line with the hypothesis of synergistic effects, previous studies showed that total extract from *G. biloba* (known for its medical applications) is more active than its isolated (poly)phenols [29, 60]. Therefore, it is reasonable to speculate that the therapeutic benefits of (poly)phenol extracts depend on the combination of its components, and on their synergistic action on diverse cellular processes. This is a rather important feature of (poly)phenol extracts in the context of multifactorial disorders, caused by a combination of genes and environmental factors and comprising the failure/impairment of several cellular pathways, such as PD.

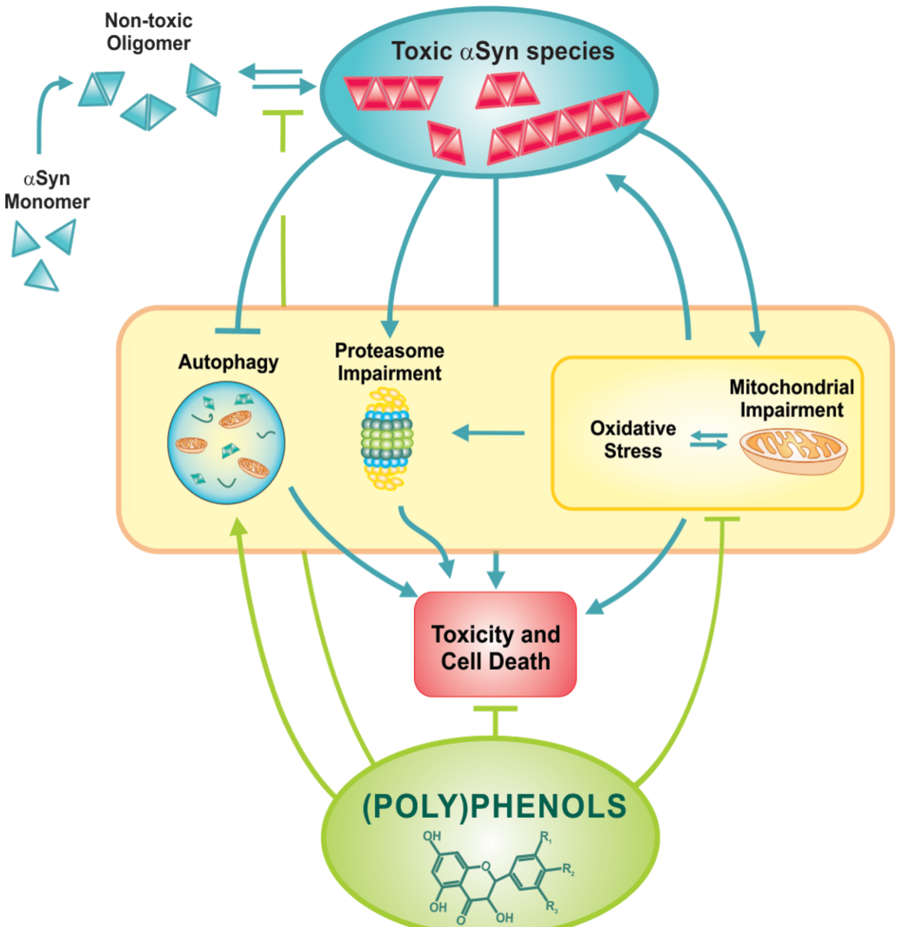


FIGURE 6. Putative mechanism of action of *C. album* leaf PEF on aSyn cytotoxicity. aSyn misfolding and aggregation promotes toxicity and cell death through disruption of several cellular functions and by increasing ROS. Mitochondria, one of the main organelles damaged, work both as a generator and a target of ROS. In turn, oxidative stress can promote aSyn aggregation by directly oxidizing it and/or indirectly by perturbing its clearance, generating a vicious cycle. Healthy cells are able to counteract aSyn-induced toxicity and aggregation by its clearance through autophagy and UPS, pathways known to be affected in pathological conditions. *C. album* leaf PEF protection could occur through (poly)phenols interaction with aSyn, inhibiting its fibrillization, and promoting the formation of stable non-toxic (protective) species, a feature of (poly)phenol observed *in vitro*. Indirectly, *C. album* leaf PEF ameliorate the general redox state of the cell, by acting as ROS scavenger and/or by promoting mitochondria function. In parallel, *C. album* leaf PEF promotes the clearance of aSyn and restores a functional autophagic flux, disrupted by aSyn toxicity. Overall, *C. album* leaf PEF modulates aSyn toxicity, leading to an increase in cell viability.

C. album leaf PEF decreased aSyn inclusion formation either by directly interacting with aSyn, stabilizing non-toxic oligomers, or by indirect mechanisms, such as through the reduction of oxidative stress and/or the stimulation of aSyn degradation pathways.

Regarding oxidative stress, we found that *C. album* leaf PEF promoted a general reduction in the levels of ROS and in particular of superoxide radical in yeast. Superoxide is the major ROS produced by the mitochondria [61, 62]. Therefore, the reduction in superoxide might be related with a more direct role of the PEF in the rescue of mitochondrial dysfunction induced by aSyn toxicity [42].

aSyn degradation occurs both via the UPS and the autophagy-lysosome pathway as reviewed in [44]. The UPS is thought to be primarily responsible for the degradation of short-lived signaling molecules or proteins tagged with ubiquitin [44]. Interestingly, overexpression of aSyn causes a decrease in proteasome function [25, 63]. However, the effects of proteasomal inhibition on aSyn degradation are still controversial. While some studies reported that aSyn appeared not to be degraded by UPS [8], others reported that proteasomal inhibition triggered accumulation and aggregation of aSyn [64].

The relevance of proteasome activity in PD is highlighted by reports that describe a decrease in the proteasome peptidase activity in the *substantia nigra* of sporadic PD patients, accompanied with a decreased expression of proteasomal α-subunits in the same brain region [4, 65]. In the conditions tested in our study, aSyn expression also led to proteasome impairment, as described before [25, 63]. Nevertheless, our results show that the mechanism responsible for the PEF protection, of aSyn cytotoxicity, is not directly related with modulation of proteasome function in the yeast PD model.

Under stress conditions, the UPS can be overwhelmed, and the autophagy pathway is then required to compensate for the increased protein accumulation and potential damage. Thus, we evaluated autophagy as a possible target of PEF bioactivity. aSyn expression inhibited the autophagic induction and flux in yeast. Recent findings relate autophagy dysfunction to aSyn-induced toxicity in a variety of PD models, from yeast to higher organisms [6, 41, 43, 66-69]. Remarkably, upon incubation with *C. album* leaf PEF the autophagy flux impaired by aSyn expression was restored. These results, together with the observation that PEF incubation reduces the percentage of cells with aSyn inclusions and

promotes its clearance, suggest that the protection promoted by this PEF may be related with increased degradation of aSyn by autophagy. In particular, recent studies indicate that the clearance of aSyn inclusions is more dependent on the autophagic pathway than on the UPS [5, 6]. We confirmed these observations in a human cell model, where we found that *C. album* leaf PEF exhibited cytoprotective properties by modulating the distribution of aSyn inclusions, protecting cells against oxidative injury induced by H_2O_2 and by promoting autophagy of aSyn expressing cells.

Our study identifies *C. album* leaf PEF as very promising agents that rescue the autophagic flux and/or decrease aSyn fibrillization resulting in the formation of non-toxic species. Our findings also suggest that PEF may increase the accessibility of aSyn inclusions to autophagic degradation. In total, our findings open a new window of opportunities for drug development, as there is accumulating evidence supporting the relevance of autophagy and aSyn modulation as a therapeutic targets in PD and other synucleinopathies [6, 10, 41, 49, 69].

Materials and methods

Plant material and extraction procedure

Fruits and leaves of *A. unedo* L. and *C. album* L. were collected by random sampling in an extensive area of Arrábida Natural Park and Comporta (southern region of Portugal), respectively. *Rubus idaeus* cv. Polka was grown in Fataca experimental field, Odemira, Portugal and *G. biloba* was grown in ITQB green house. Plant material was extracted as described in [70].

Extract fractionation by solid phase extraction

Hydroethanolic extracts were fractionated by solid phase extraction (SPE) as described in[35].

Total phenolic quantification

Determination of total phenolic compounds was performed by the Folin-Ciocalteau method adapted to microplate reader [71].

Peroxyl radical scavenging capacity determination

Peroxyl radical scavenging capacity was determined by the ORAC (Oxygen Radical Absorbance Capacity) method as described [28].

Phenolic profile determination by LC-MS

(Poly)phenol fractions were applied to a C-18 column (Synergi Hydro C18 column with polar end capping, 4.6 mm x 150 mm, Phenomenex®) and analysed on a LCQ-DECA system controlled by the XCALIBUR software (2.0, ThermoFinnigan), as described [28].

Yeast strains, transformation and plasmids

Four strains of *Saccharomyces cerevisiae* were used in this study. BY4741 (*MAT*a; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) and its isogenic mutants *Δsod1* (*MAT*a; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *Δsod1::KanMX4*) and *Δyap1* (*MAT*a; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0* *Δyap1::KanMX4*) were acquired from Euroscarf (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/) and carry a *KanMx4* deletion cassette replacing the gene in question. The AD1-8 strain has eight deletions of so called multidrug resistance (MDR)

genes (*Δyor1*, *Δsnq2*, *pdr5-Δ2*, *Δpdr10*, *Δpdr11*, *Δycf1*, *pdr3-Δ2*, *Δpdr15*) encoding for ABC transporters involved in xenobiotics efflux [72]. Yeast cells were transformed with the indicated plasmids using lithium acetate standard method [73]. The empty plasmids p426GAL, p413GPD or pRS415 were used as control [74]. The p316-GFP-*ATG8* plasmid [45] was a kind gift from Prof Yoshinori Oshumi (National Institute for Basic Biology, Okazaki, Japan) and was used to sub-clone GFP-*ATG8* as well as the endogenous *ATG8* promoter into the *SacI* - *XhoI* sites of pRS415 plasmid [74]. Other plasmids used in this study were previously described: p425GAL-aSyn-GFP, p426GAL-aSyn-GFP and p426GAL-aSyn plasmids carrying the human gene of aSyn with or without a C-terminal fusion to GFP under the regulation of *GAL1* inducible promoter [25]; p316-2xmCherry-*ATG8*, expressing Atg8 with an N-terminal fusion to mCherry under the regulation of the endogenous *ATG8* promoter (a kind gift from Dr. Kuninori Suzuki, Tokyo Institute of Technology)[75]; p413GPD-GFPu expressing an unstable GFP under the regulation of *GPD* constitutive promoter.

Yeast growth conditions and compound testing

A pre-inoculum was prepared until the optical density at 600 nm ($OD_{600\ nm}$) reached approximately 0.5±0.1 (*log* growth phase) in synthetic complete (SC) medium [0.67% (w/v) yeast nitrogen base without amino acids (Difco), 2% (w/v) glucose and 0.79 $g.L^{-1}$ complete supplement mixture (CSM) (QBiogene)], under shaking at 30 °C. Afterwards cells were diluted in the same medium to obtain all strains in *log* growth phase. Readings were performed in a 96 well microtiter plate using a plate spectrophotometer (Biotek Power Wave XS). To determine the *lag* and doubling time the strains were grown in SC medium, as described before, and diluted to an $OD_{600\ nm}$ of 0.03 in the same medium supplemented with 250, 500 or 1000 μg GAE. mL^{-1} of the indicated PEF in a 96 well microtiter plate. The cells were then incubated at 30 °C with shaking for 24 h. Yeast growth was kinetically monitored hourly during 24 h by measuring the $OD_{600\ nm}$ and the growth curves were obtained. For each treatment doubling and *lag* phase times were determined based on the linear regression of the *log* growth phase equation, obtained after logarithmic transformation of $OD_{600\ nm}$ values. For spot assays with H_2O_2 the strains were grown as described and afterwards were inoculated ($OD_{600\ nm}$ 0.03) in SC medium with 1000 μg GAE. mL^{-1} of PEFs. After 6 h of

growth cells were pelleted and resuspended in SC medium supplemented with 2 mM H_2O_2. Subsequently, 30 µL of cells were withdrawn and $OD_{600\ nm}$ was adjusted to 0.05±0.005 with SC medium. Serial dilutions were made with a ratio of 1:4 and 4 µL of each dilution was spotted in YPD solid medium and grown for 2 days at 30 °C.

For growth curves with yeast cells expressing aSyn, cells were grown in the appropriated SC selective liquid medium containing 1% (w/v) raffinose, until *log* phase was reached. Subsequently, cells were incubated for 6 h at 30 °C with shaking on the same medium not supplemented or with PEFs. Afterwards, cells were centrifuged and diluted to $OD_{600\ nm}$ 0.03 in new SC selective medium 2% (w/v) glucose (Control, aSyn OFF) or 1% (w/v) galactose (aSyn ON) not supplemented or with PEFs, and grown for 24 h at 30 °C with shaking. The growth was kinetically monitored each hour by measuring $OD_{600\ nm}$.

For aSyn cytotoxicity evaluation spot assays were performed on solid medium using cells treated with PEFs as described above. $OD_{600\ nm}$ was set to 0.05±0.005 and 1:4 serial dilutions of each sample were prepared. Then, 4 µL of each dilution was spotted in solid SC selective medium containing 2% (w/v) glucose (aSyn OFF) and incubated at 30 °C for 42 h. Images were acquired using Chemidoc™ XRS and Quantity-one® software. To evaluate cells viability by flow cytometry with PI, cells were subjected to the previously described treatment with *C. album* leaf PEF (0.5 to 30 µg GAE. mL^{-1}) or the pure compounds (0.5 to 30 µg. mL^{-1}), myricetin-3-*O*-galactoside, myricetin-3-*O*-glucoside or quercetin-3-*O*-galactoside (Extrasynthese, Genay Cedex, France). Data analysis was performed using FlowJo software and the cell doublets exclusion was performed based on Forward-A and -W scatter parameters. A minimum of 10000 events were collected for each experiment.

For aSyn clearance experiments, after 12 h of aSyn expression induction the cells were centrifuged washed in PBS, resuspended in 2% (w/v) glucose SC liquid media (aSyn expression OFF) and incubated at 30 °C, with shaking, for 24 h. The levels of aSyn were determined by western blotting at 12 h of induction (corresponding to 0 h of clearance) and at 24 h of clearance.

Yeast metabolic capacity assay

Yeast cells were grown as described above. The growth was kinetically monitored each hour by measuring $OD_{600\,nm}$ and metabolic capacity was assessed at 6 h of growth using CellTiter-Blue® Cell Viability assay (Promega), following manufacturer instructions.

H4 cell culture and transfections

Human H4 neuroglioma cells (gift from Dr. Bradley T. Hyman, Harvard Medical School) were maintained at 37 °C in OPTI-MEM I (Gibco, Invitrogen, Barcelona, Spain) supplemented with 10% (v/v) fetal bovine serum and seeded at 80,000 cells.cm^2 density 24 h prior to transfection. Cells were transfected as previously described [76] with pcDNA3.1-aSynT and pcDNA3.1-Synphilin-1-V5.

Compound testing in H4

For H_2O_2 toxicity assay cells were treated with 30 μg GAE. mL^{-1} *C. album* leaf PEF for 16 h, the medium was removed and cells were treated with 600 μM H_2O_2 for 6 h. To evaluate the effect of PEFs in aSyn aggregation, 24 h post transfection cells were treated with 30 μg GAE. mL^{-1} *C. album* leaf PEF for 6 h.

Western blotting

For aSyn quantification total yeast protein extraction was performed by glass bead lysis as described before [77, 78]. For GFP-Atg8 quantification, protein extraction was performed using the method described by Cheong *et al*, 2005 [79]. For GFP-Atg8 quantification, cells were treated with 10% (v/v) trichloroacetic acid (TCA), and washed twice with acetone. The dry cell pellet was then resuspended in MURB buffer (50 mM Na_2HPO_4, 25 mM MES, pH 7.0, 1% (v/v) SDS, 3 M urea, 0.5% (v/v) 2-mercaptoethanol, 1 mM NaN_3, and 0.05% (w/v) bromophenol blue) with proteases and phosphatases inhibitors (Roche, Mannheim, Germany), and disrupted by vortex with an equal volume of acid-washed glass beads, for 5 min. The samples were incubated at 70 °C for 10 min and cell debris were removed by centrifugation. After extraction, western blot was performed following standard procedures. Using the GFP-Atg8 processing assay *ATG8* induction and autophagic flux were determined [80]. *ATG8* induction was quantified by the determination of the fold increase of total GFP

signal (GFP-Atg8 and free GFP signal, detected with anti-GFP) normalized to PGK; autophagic flux was quantified by measuring the vacuolar degradation of the Atg8 domain reporter (ratio of free GFP to total GFP signal) [81].

For aSyn quantification in human H4 neuroglioma cells were lysed with NP-40 lysis buffer in the presence of protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany), western blot was performed following standard procedures. Autophagy was quantified as the accumulation of the autophagosome-associated protein LC3. Cells were treated with 20 mM NH_4Cl and 200 µM leupeptin for 2 h or 4 h, followed by immunoblotting for LC3. Flux was calculated as the ratio of treated (4 h) to untreated samples after normalization to the loading control, adapted from [46, 50]. Basal autophagy and autophagic flux results are presented as the ratio of PEF treated and vehicle treated cells.

Antibodies used: aSyn (BD Transduction Laboratories, San Jose, CA, USA), GAPDH (Ambion, Cambridgeshire, UK), GFP (Antibodies Incorporated, Davis, CA, USA), PGK (Life Technologies, Paisley, UK), LC3 (nanoTools, Teningen, Germany), β-actin (Abcam, Cambridge, UK).

Flow cytometry (FCM)

FCM was performed in a FACS BD LSR Fortessa, equipped with the *695/40 BP and the 685 LP*. To determine the levels of ROS, yeast cells were incubated with 50 µM DCFHDA (Molecular Probe, Life Technologies) or 30 µM DHE (Molecular Probe, Life Technologies), for 15 min at 30 °C, with agitation and protected from light [39]. To analyze cell viability with PI, yeast or H4 cells were incubated with 5 $\mu g.mL^{-1}$ of PI, for 30 min protected from light. To study the proteasome impairment, yeast cells were transformed with the aSyn and GFPu encoding plasmid or the respective empty plasmid. Autophagy was analyzed in cells transformed with the mCherry-Atg8 and aSyn-GFP encoding plasmids or with the empty plasmids in a BD FACSAria, cells treated with rapamycin were used as control. Data analysis was performed using FlowJo software and the exclusion of cell doublets was performed based on Forward-A and -W scatter parameters. A minimum of 10000 events were collected for each experiment. Results were expressed as median fluorescence intensity (MFI) of a molecule.

Fluorescence microscopy

In order to determine the percentage of yeast cells with aSyn inclusions, cells were grown as described above and GFP fluorescence was visualized using a Leica DM3000 fluorescence microscope. The proportion of cells presenting aSyn inclusions was then determined by counting at least 800 cells for each treatment using ImageJ software. Transfected H4 cells were fixed and permeabilized with methanol and blocked in 1.5 % (v/v) normal goat serum in PBS for 1 h. Cells were incubated with primary antibody overnight at 4 °C (mouse anti-aSyn; BD Transduction Laboratories, San Jose, CA, USA) followed by secondary antibody incubation for 1 h (goat anti-mouse IgG-Alexa488, Invitrogen Corporation, Carlsbad, CA, USA). Slides were subjected to fluorescence microscopy with a Zeiss Axiovert 200 M Widefield Fluorescence microscope. The proportion of cells with aSyn inclusions within the population was then determined by counting at least 100 cells per condition using ImageJ software [24].

ThioflavinT assay

The expression and purification of human aSyn was performed as described in [76]. For the thioflavin T (ThT) assay, the protein solution (70 µM) was mixed with *C. album* leaf PEF to a final concentration of 0.5 or 30 µg GAE. mL^{-1} in 50 mM Tris-HCL buffer (pH 7.4). Protein samples were stirred at 900 rpm, 37 °C in a thermomixer. 1.4 µM of aSyn was added to 1 µM of ThT solution in 50 mM Tris-HCL buffer and ThT fluorescence was recorded at the tested time-points as in [82]. The samples obtained in the different time-points were analyzed by SDS-PAGE using standard western blotting procedures as described above. The toxicity of aSyn species formed at 0 h and 48 h of fibrillization was evaluated as described in [83]. Cells treated only with the correspondent concentration of *C. album* leaf PEF were used as controls. Cells treated with 10 % (v/v) Triton X-100 for 10 min, were used for normalization.

Triton soluble and insoluble fractions

Total protein was extracted and quantified with the BCA protein assay kit (Thermo Fisher Scientific Inc, Illinois, USA). Total protein (200 µg) was incubated with 1% Triton X-100 on ice, for 30 min. Protein fractions were separated by centrifugation at 15000 g for 60 min at 4 °C. The top soluble protein fraction (T-Soluble) was collected and the insoluble protein

fraction (T-Insoluble) pellet was resuspended in 40 µL of 2% SDS Tris-HCl buffer pH 7.4 by pipetting and subsequent sonication (10 seconds). Total protein, T-Soluble and T-Insoluble fractions (5 µl of each) were loaded and resolved by SDS-PAGE, western blotting was performed as previously described.

Statistical analysis

The results reported in this work are the average of at least three independent biological replicates and are represented as the mean ± SD. Differences among treatments were assessed by analysis of variance with Tuckey HSD (Honestly Significant Difference) multiple comparison test ($\alpha = 0.05$) using SigmaStat 3.10.

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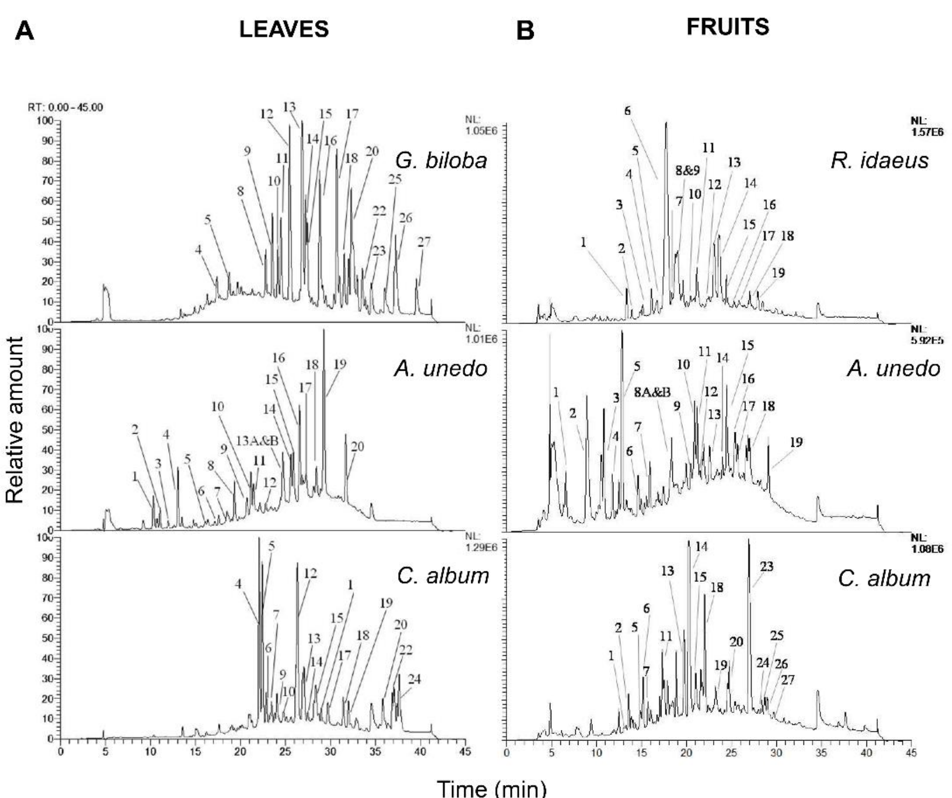
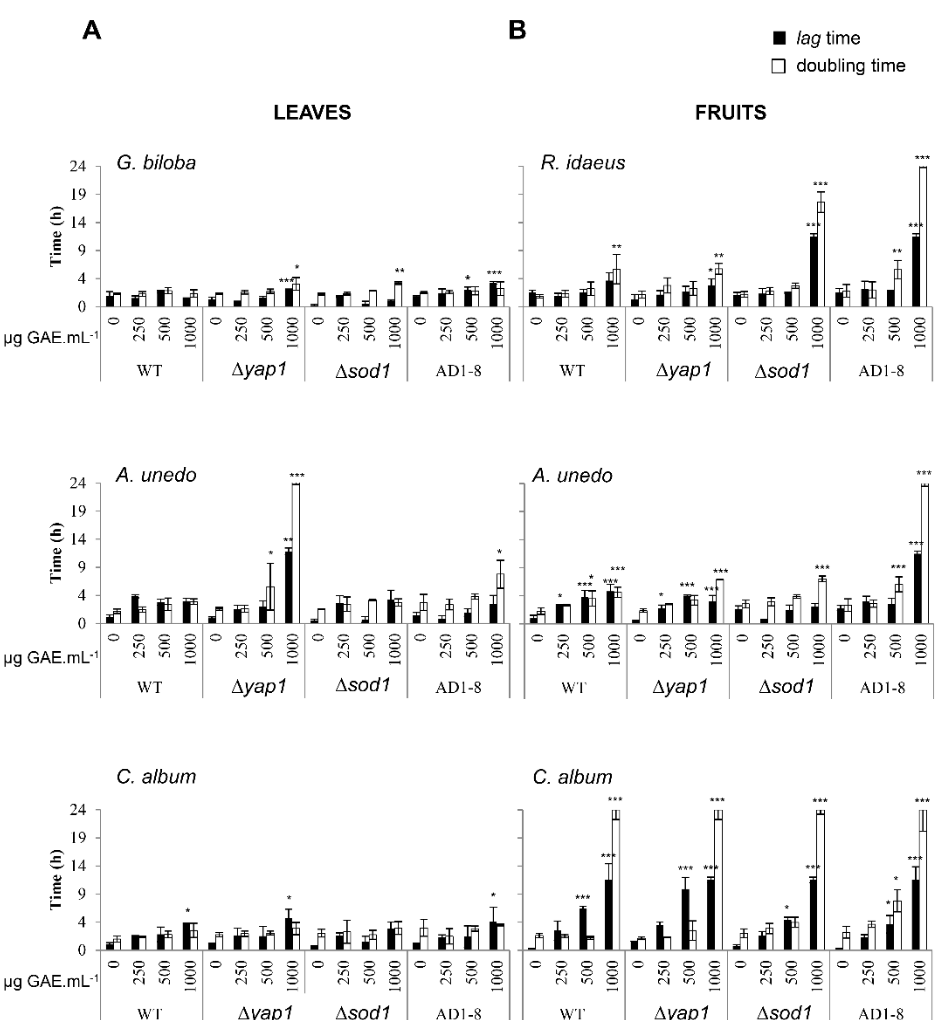
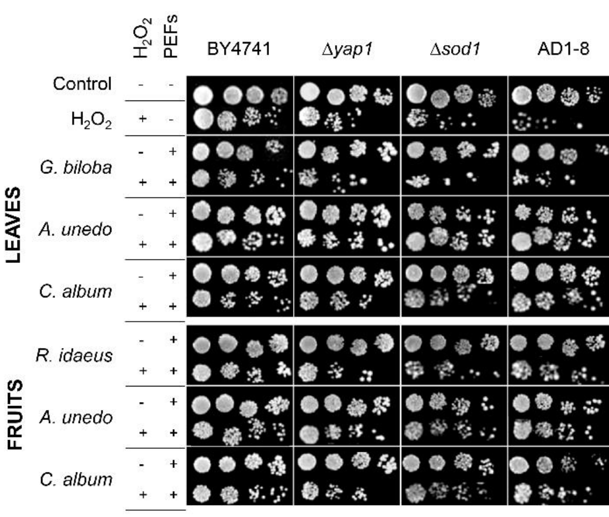
Supplementary material



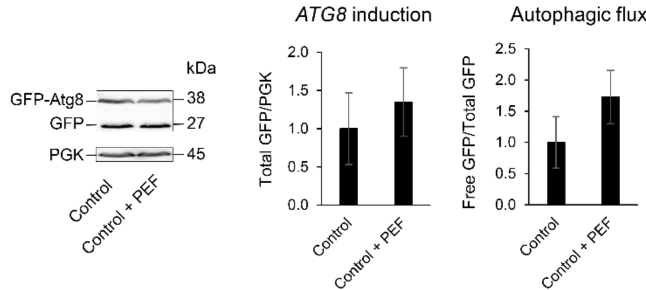
SUPPLEMENTAL FIGURE 1. HPLC profile of PEFs recorded at the maximum absorption wavelength. Chromatograms for A) leaves or B) fruits PEFs are presented. The full scan deflection is shown in the upper right corner of each panel. Numbers correspond to peaks putative identification based on MS fragmentation pattern, in comparison with bibliography presented in Table S1 to S6. Values are expressed in arbitrary units.



SUPPLEMENTAL FIGURE 2. PEFs differentially affect the *lag* time and doubling time of BY4741 (WT), *Dyap1, Dsod1* and AD1-8 *S. cerevisiae* strains. Yap1 is a transcription factor that regulates gene expression of SOD1 (encoding the cytosolic copper-zinc superoxide dismutase), as well as many other genes involved in the oxidative stress response in yeast. AD1-8 cells have 8 deletions in genes that encode for xenobiotic efflux transporters. Cells were treated for 24 h with the indicated concentrations of PEFs from **A)** leaves or **B)** fruits. *Lag* (■) and doubling time (□) acquired at the maximal growth rate, obtained from the growth curves. Values represent the mean ± SD of three independent growth curves. Statistically significant different results from the control without treatment with PEFs are indicated, *p<0.05, **p<0.01 and ***p<0.001.



SUPPLEMENTAL FIGURE 3. PEFs increase the viability of *S. cerevisiae* strains subjected to oxidative stress. Spot assay of the indicated yeast strains treated with PEFs for 6 h in glucose medium and subjected to 2 mM H_2O_2 for 1 h. Each experiment was performed at least three times and a representative result is shown.



SUPPLEMENTAL FIGURE 4. Autophagy in control cells treated with PEF. BY4741 cells transformed with the empty vector (control cells) were treated with C. album leaf PEF for 6 h in raffinose medium, prior to treatment for 12 h with PEF in galactose medium. A) Autophagy was evaluated by GFP-Atg8 processing assay assessed by western blot (left panel). ATG8 induction quantified by the total GFP signal (GFP-Atg8 and free GFP signal, detected with anti-GFP) (middle panel); autophagic flux quantified by measuring the vacuolar degradation of the Atg8 domain reporter (ratio of free GFP to total GFP signal) (right panel). Values represent the mean ± SD of three independent experiments.

Table S1. Peaks assignments, retention times and mass spectral data of phenols present in *G. biloba* PEF

Peak No.	RT	PDA	M/Z [M-H]	MS^2	Putative identity
1	14.92	235	609.2, 593.2, 441.2, 305.2	483.1, 441.0, 432.0, **305.1**	Epigallocatechin dimer
2	15.54	275	255.1	193.0, 165.1	Unknown
3	16.75	275	913.2, 745.2, 699.3, 537.3, 449.4, 375.3, 231.2	multiple	Unknown
4	17.4	275	**787.2**, 641.1, 593.2, 478.3, 371.1, 315.3	625.1, 478.2, 315.2	Isorhamnetin-3-*O*-rhamnosylhexoside-7-*O*--glucoside (1, 2)
5	18.76	270	**771.2**, 625.3, 462.3, 305.2, 299.3	**609.2**, 463.1, 301.2	Quercetin-3-*O*-rhamnosylhexoside-7-*O*-glucoside (1)
6	19.69	275	**729.2**, 595.1, 489.1	multiple	Unknown
7	21.32	275	**449.3**, 287.3	421.0, 287.1, 259.0	Unknown flavonoid hexoside (3)
8	22.82	355	**755.1**, 301.3	593.2	3-*O*-[2-*O*,6-*O*-Bis(α-L-rhamnosyl)]-β-D--glucosyl]quercetin (4)
9	23.55	355	625.3, 316.3	316.2	3-*O*-[6-*O*-(α-L-rhamnosyl)-β-D--glucosyl]isorhamnetin (4)
10	24.14	265sh, 350	**739.2**, 285.3	285.2	3-*O*-[2-*O*,6-*O*-Bis(α-L-rhamnosyl)]-β-D--glucosyl]kaempferol (4)
11	24.5	265sh, 355	479.3, 316.3	316.2	3-*O*-(β-D-Glucosyl)isorhamnetin (4)
12	25.45	270sh, 350	609.3, 301.3	301.1	3-*O*-[6-*O*-(α-L-rhamnosyl)-β-D--glucosyl]quercetin (4)
13	26.85	345	463.2, 301.3	301.1	Quercetin-3-*O*-hexose (1)
14	27.25	265, 345	**593.3**, 285.3	285.1	3-*O*-[6-*O*-(α-L-rhamnosyl)-β-D--glucosyl]kaempferol (4)
15	27.43	350	**623.3**, 315.1; **593.3**, 301.1	316.1, 301.1	Myricetin-3-*O*-rutinoside or isorhamnetin-3-*O*--rutinoside or isorhamnetin-3-*O*-

Chapter 2

Table S1. *Continuation*

					-neohesperidoside or isorhamnetin-3-*O*--2"glucosylrhamnoside (1)
16	28.81	265, 345	**879.2+439.2**, 593.3, 447.3, 285.3		Ginkgolide C, Kaempferol rutinoside and kaempferol hexose (1)
17	30.7	265, 315, 355	**755.3**, 301.3, 271.4, 255.4	609.1, 301.1	Quercetin-3-*O*-2"-(6"-*p*--coumaroyl)glucosylrhamnoside (1)
18	31.53	255, 350	**755.3**, 301.3, 271.4, 255.4	609.1, 301.1	Quercetin-3-*O*-2"-(6"-*p*--coumaroyl)glucosylrhamnoside (1)
19	32.02	265, 345	**747.3**, 301.3	multiple	Quercetin-3-*O*-p-coumaroylgdiglycoside (1)
20	32.31	265, 315	**739.1**, 285.3	593.1, 285.1	3-*O*-[2-*O*,6-*O*-Bis(α-L-rhamnosyl)]-β-D--glucosyl]kaempferol (4)
21	32.97	265, 340	**739.1**, 300.3, 285.3	593.1, 285.1	3-*O*-[2-*O*,6-*O*-Bis(α-L-rhamnosyl)]-β-D--glucosyl]kaempferol (4)
22	33.54	265, 345	**739.2**, 301.3, 285.3	593.1, 285.1	3-*O*-[2-*O*,6-*O*-Bis(α-L-rhamnosyl)]-β-D--glucosyl]kaempferol (4)
23	34.54	265sh, 315	**739.2**, 301.3, 285.3	593.1, 285.1	3-*O*-[2-*O*,6-*O*-Bis(α-L-rhamnosyl)]-β-D--glucosyl]kaempferol (4)
24	34.9		423, 847	423.0, 367.1	Gingkolide B (1)
25	36.04	270, 330	551.2	519.3, 457.2, 289.1	Bilobetin or sequoiaflavone (1)
26	37.23	270, 325	**565.3**, 533.4, 311.5	533.2, 389.2	Ginkgetin or isoginkgetin (4)
27	39.53	270, 325	579.3	547.2, 403.2	Sciadopitysin (1)

The most abundant ions are shown in bold. Numbers in brackets are references.
Sh indicates a shoulder presented in the PDA chromatogram.

Table S2. Peaks assignments, retention times and mass spectral data of phenols present in *A. unedo* leaves PEF

Peak No.	RT	PDA	M/Z [M-H]	MS^2	Putative identity
1	10.33	280	317.2	271.0, 161.0, 109.2	Unknown
2	11.07	280	331.1	271.1, 211.1, 169.1	Gallic acid glucoside (2)
3	11.97	270	**331.2**, 271.4, 169.1	271.1, 169.1	B-D-Glucogallin or gallic acid 4-*O*-[illegible]-D- -glucopyranoside (5)
4	13.05	275	**343.2**, 191.1	191.2, 169.2	Galloylquinic acid (6)
5	16.09	275	**593.1**	467.1, 425.1, 407.3, **289.1**	Prodelphinidin dimer
6	17.57	275	**761.3**, 731.4, 593.3, 465.3, 439.5, 371.3	**609.1**, 591.2, 423.0, 305.1	Prodelphinidin dimer gallate
7	18.05	280	**577.1**, 425.3, 289.3	425.1, 407.3, **289.2**	Procyanidin dimer B2 (2)
8	19.32	275	423.1	313.2, **169.1**	Gallotannin (5)
9	20.73	280	289.2	245.2	Catechin (2)
10	21.15	275	**477.1**, 325.3	325.3	Digalloyl shikimic acid (5)
11	21.46	275	**633.1**, 463.1, 301.4, 275.3	463.1, **301.2**	Strictinin ellagitannin (2)
12	22.82	275	**729.1**, 635.1, 477.1	**577.1**, 559.2, 451.2, 425.2, 407.3	Procyanidin dimer gallate (7)
13A	24.73	260-360	479.3	317.2	Myricetin glucoside (5)
13B	24.73	260-360	**433.3**, 301.4	301.3	Quercetin arabinoside or xyloside (2)
14	25.61	265-355	**615.1**, 301.3	**463.2**, 301.2	Quercetin hexose galloyl derivative (2)
15	25.87	265-355	**615.3**, 463.3, 300.2	**463.2**, 301.2	Quercetin hexose galloyl derivative (2)
16	26.56	265-350	**463.2**, 317.3	317.3	Myricetin rhamnoside (5)

Table S2. *Continuation*

17	27.26	275-350	**463.3**, 301.1	301.1	Quercetin-3-*O*-glucoside (2)
18	28.45	270-350	**599.1**, 285.1	**447.2**, 313.2, 285.2	Kaempferol galloylglucoside (5)
19	29.26	340	**447.2**, 301.3	301.2	Ellagic acid rhamnoside (2)
20	31.70	265, 345	**431.2**, 285.2	285.2	Kaempferol rhamnoside (5)

The most abundant ions are shown in bold. Numbers in brackets are references.

Table S3. Peaks assignments, retention times and mass spectral data of phenols present in *C. album* leaves PEF

Peak No.	RT	PDA	M/Z [M-H]	MS^2	Putative identity
1	16.23	280	**451.2**, 289.1	289.1	Catechin glucoside
2	17.64	285	371.1	325.1, 163.0	*p*-coumaric acid hexose
3	20.94	280	289.3	245.4	Catechin (2)
4	22.09	260, 350	**479.3**, 317.2	317.1	Myricetin-3-*O*-galactoside (8)
5	22.42	275, 350	**479.2**, 317.2	317.2	Myricetin-3-*O*-glucoside (8)
6	22.87	275	289.2	245.1, 205.1	Epicatechin (2)
7	23.45	275, 350	**463.1,** 317.1	317.1	Myricetin rhamnoside (5)
8	23.82	280	**863.2**, 289.2	multiple	Procyanidin trimer
9	24.07	275, 325	**463.3**, 301.2	301.2	Quercetin-3-*O*-glucoside or 3-galactoside (2, 8)
10	24.8	280	**1151.2**, 863.3	multiple	Procyanidin tetramer
11	25.11	280	863.2	multiple	Proanthocyanidin trimer
12	26.33	260, 350	**493.2**, 331.2, 317.2	331.1, 316.9	Methylquercetin hexose
13	27.07	355	**463.3**, 301.3	301.1	Quercetin-3-*O*-galactoside (2)
14	27.47	275	**463.3**, 301.3	301.1	Quercetin-3-*O*-glucoside (2)
15	28.38	275	1151.1	multiple	Proanthocyanidin tetramer
16	28.97	265, 345	**447.3**, 285.1	285.1	Kaempferol-3-*O*-glucoside (9)
17	29.72	280	861.2	709.1, 423.1,285.1	Proanthocyanidin gallate
18	31.41	270	**535.2**, 241.1	241.1	Stilbene derivative (10)
19	32	285	417.3	255.1, 211.0	Stilbene hexose
20	35.82	275	317.2, 301.2	ND	Unknown

Table S3. *Continuation*

21	36.87	285	255.1	255.1, 213.1, 136.0	Stilbene
22	37.06	290	255.1	255.1, 213.1, 136.0	Stilbene
23	37.39	275, 310	311.1	311.1, 183.2	Unknown
24	37.64	350-280	301.2	283.1, 245.0	Ellagic acid or quercetin (2, 11)

The most abundant ions are shown in bold. Numbers in brackets are references. ND, not detected.

Table S4. Peaks assignments, retention times and mass spectral data of phenols present in *R. idaeus* fruits PEF

Peak No.	RT	PDA	M/Z [M-H]	MS²	Putative identity
1	13.34	280	789.3	multiple	Ellagitannin derivative
2	13.96	285	759.2	multiple	Ellagitannin derivative
3	15.14	280	**343.3**, 163.3	163.1	Galloylquinic acid (6)
4	16.09	260	**783.2**, 481.3, 301.2	481.1, 301.2	Bis-hexahydroxydiphenic acid-glucose or pedunculagin isomer (12, 13)
5	16.57	375, 530	**899.2**, 575.3, 287.3 (+)	Multiple (+)	Cyanidin conjugate
6	17.75	270, 515	**611.1**, 287.3 (+)	287.2 (+)	Cyanidin-3-*O*-sophoroside (2)
7	18.36	515	**783.2**, 301.2	481.2, 301.2	Bis-hexahydroxydiphenic acid-glucose or pedunculagin isomer (12, 13)
8	18.96	280	**1567.1,** 1250.9, 301.2	1265.0, 1235.1, 933.1	Sanguiin-H10 isomer (14)
9	20.38	280	**1717.1**	multiple	Sanguiin-H6 without gallic acid
10	18.76	275 515	**595.1**, **449.1**, 287.3 (+)	287.3	Cyanidin-3-glucoside + cyanidin-3-rutinoside (2)
11	21.15	240, 275	**577.2** / **1567.1**, 783.4,	multiple	Procyanidin dimer or Sanguiin H-10 isomer (14)
12	22.47	275	289.2	245.1, 205.1	Epicatechin (2)
13	23.04	280	**1401.2**, 934.4, 301.2	1869.2, 1567.1, 1250.2	Lambertianin C (2)
14	23.63	280	**1869.1**, 1567.1, 1215.2, 934.5, 561.2, 301.4	1567.1, 1265.1, 1235.1	Sanguiin-H6 (2)
15	24.43	360	**433.3**, 301.4	301.2	Ellagic acid pentoside
16	25.29	360	**433.3**, 301.4	301.2	Ellagic acid pentoside
17	25.82	275	**935.3**, 301.2	633.1, 301.1	Ellagitannin derivative
8	27.02	365	301.2	301.2, 275.1	Ellagic acid (2)
19	27.91	360	447.3, 315.1	315.1	Methyl ellagic acid conjugate (2)

The most abundant ions are shown in bold. Numbers in brackets are references.
(+) represent masses detected in the positive mode.

Table S5. Peaks assignments, retention times and mass spectral data of phenols present in *A. unedo* fruits PEF

Peak No.	RT	PDA	M/Z [M-H]	MS^2	Putative identity
1	6.58	290	1151.1	863.1	Proanthocyanidin trimer
2	8.87	290	389.2	209.1	Unknown
3	10.82	280	331.3	271.1, 211.1, **169.1**	Gallic acid glucoside (2)
4	11.76	265	331.3	271.1, **169.1**	Galloyl glucoside (2)
5	12.83	265	343.2	191.2	3-*O*- or 5-*O*-galloylquinic acid (6)
6	14.61	255-300	**331.2**, 191.3	169.1	Gallic acid 4-*O*-β-D-glucopyranoside or β-D-glucogalline (6)
7	15.93	255-300	325.3	169.1	Galloyl shikimic acid (5)
8A	18.35	280, 515	**449.2**, 287.1 (+)	287.1 (+)	Cyanidin-3-*O*-hexose (11)
8B	18.35	280, 515	495.1	343.1, **191.0**	Digalloyl quinic acid (5)
9	20.46	280	289.2	245.3	Catechin (2)
10	20.9	275	477.1	325.1	Digalloyl shikimic acid (2)
11	21.18	280	**633.1**, 477.1, 401.0, 387.4, 301.4, 289.3	463.1, 301.3, 275.3	Strictinin ellagitannin (5)
12	21.78	280-360	**635.2**, 463.3, 301.4	**463.3**, 301.2	Quercetin hexose galloyl derivative (2)
13	22.56	280	1109.2, 727.9, 689.2, 647.2, 539.4, 477.2, 301.2	multiple	Gallotannin derivative (5)
14	23.99	280	366.3	204.1, 186.1, 142.3	Unknown (2)
15	24.47	280-360	**433.1**, 301.4	301.4	Ellagic acid arabinoside or xyloside (2)
16	25.39	275-350	**615.1**, 433.3, 300.4	463.2, **301.2**	Quercetin hexose galloyl derivative (2)
17	25.66	275-355	**615.1**, 301.3	463.1, **301.2**	Quercetin hexose galloyl derivative (2)
18	27.02	275-350	**463.1**, 301.2	301.3	Quercetin-3-glucoside (2)
19	29.09	275	**447.1**, 301.3	301.3	Ellagic acid rhamnoside (5)

The most abundant ions are shown in bold. Numbers in brackets are references. (+) represent masses detected in the positive mode.

Table S6. Peaks assignments, retention times and mass spectral data of phenols present in *C. album* fruits PEF

Peak No.	RT	PDA	M/Z [M-H]	MS²	Putative identity
1	13.29	280	**371.2**, 353.1,191.2	191.2	Hydroxycinnamic acid derivative
2	13.58	280	**371.2**, 191.2	341.1, 191.2	Hydroxycinnamic acid derivative
3	13.88	275	**533.2**, 339.3, **191.2**	353.0, 191.1	Chlorogenic acid derivative
4	14.04	280	**371.2**, 191.2	341.1, 191.2	Hydroxycinnamic acid derivative
5	14.88	285	**421.2**, 333.2, 191.2	333.0, 171.0	Unknown
6	15.18	250, 290	**315.3**	153	Dihydroxybenzoic acid hexose
7	15.71	290, 325	**515.3**, 191.1	**353.1**, 191.2	Dicaffeoyl quinic acid (15)
8	16.75	285	**707.2**, 679.3, **353.2**, 191.2	353.2	Chlorogenic acid derivative
9	17.03	280	**707.2**, 191.2	353.2	Chlorogenic acid derivative
10	17.31	290, 325	**353.2**, 191.2, 179.2	191.2	Chlorogenic acid derivative
11	17.54	285	707.2	515.3, 463.1,323.9	Unknown
12	18.46	275	**707.2**, 533.3, 515.4, 191.2		Chlorogenic acid derivative
13	19.73	290, 310	**533.3**, 353.3, 341.3, 191.3, 177.9	489.1, 359.0, 191.1	Unknown
14	20.2	315	**353.1**, 191.3	191.2	Chlorogenic acid derivative
15	21.02	280	**355.2**, 315.4, 193.2	193.1	Ferulic hexose
16	21.58	280, 330	335.1, 183.2	183.2, 169.1	Unknown

Table S6. *Continuation*

17	21.76	280, 310	341.3, 179.2	179.2	Hydroxycinnamic acid-hexose
18	22	315	**353.1**, 191.3	191.2	Chlorogenic acid derivative
19	23.23	280	**513.3**, 401.3, 289.3	401.1, 289.1	Catechin derivative (2)
20	24.73	265, 355	**479.3**, 317.3	317.2	Myricetin-3-*O*-hexoside (16)
21	25.4	280	**567.2**, 521.3, 515.4, 319.4	341.2, 329.3	Unknown
22	26.36	275	493.3	331.1	Methylquercetin hexose
23	26.92	350	**463.2**, 301.3	301.2	Quercetin-3-*O*-hexoside (16)
24	28.36	280	575.3, 423.3	multiple	Proanthocyanidin dimer
25	28.7	270, 355	**433.3**, 301.3	301.3	Quercetin-3-*O*-pentoside (16)
26	28.94	270	**447.3,** 285.2	285.2	Kaempferol hexose (16)
27	29.68	280	861.2	709.1, 575.0, 423.1	Proanthocyanidin derivative
28	30.84	280	755.3, 301.1	**609.2**, 301.1	Quercetin derivative
29	31.38	280	**535.4**, 241.4	241.1	Stilbene hexose (10)
30	32.58	280	739.4, **451.3**, 341.3	ND	Unknown
31	37.01	285	255.1	255.1, 213.1, 211.1, 151.1	Stilbene

The most abundant ions are shown in bold. Numbers in brackets are references.

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Chapter 3

(Poly)phenol-digested metabolites protect against aSyn toxicity

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This chapter is based on the following manuscript:

(Poly)phenol-digested metabolites modulate alpha-synuclein toxicity by regulating proteostasis

Macedo D, Jardim C, Figueira I, MacDougall GJ, Stewart D, Ferreira RB, Tenreiro S, Outeiro TF and Santos CN *(manuscript submmited).*

This chapter contains data in which the author of this dissertation executed the majority of the experiments. Jardim C. determined the GSH/GSSG and Figueira I the MS data analysis.

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Abstract

Parkinson's disease (PD) is an age-related neurodegenerative disease that is associated with the misfolding and aggregation of alpha-synuclein (aSyn). Nutrition plays an important role in the prevention, onset, and progression of this disease, and dietary (poly)phenols are known to revert and prevent age-related cognitive decline and neurodegeneration in various model systems. However, only limited attempts were done to evaluate the impact of digestion on the bioactivities of (poly)phenols, and their underlying mechanisms of action remain unclear. This constitutes a challenge for the design of guidelines for (poly)phenol-based therapies. Here, (poly)phenols from *Arbutus unedo* (leaves and fruits) were subjected to an in vitro digestion procedure, and afterwards tested in PD models in order to evaluate their mechanisms of action.

The (poly)phenol-digested metabolites from A. unedo leaves effectively counteracted aSyn and H_2O_2 toxicity in yeast and human cells, improving viability by reducing aSyn aggregation and by inducing its clearance pathways, autophagy and proteasome. In addition, these metabolites modulated pathways known to be associated with aSyn toxicity such as oxidative stress, endoplasmic reticulum (ER) stress, mitochondria impairment and SIR2 expression.

This pioneering study pinpoints the molecular mechanism of (poly)phenol-digested metabolites in PD revealing a protective pleiotropic role in several of the known pathways of aSyn toxicity. Overall, (poly)phenols reduce aSyn toxicity, enhance the efficiency of the ER-associated degradation, by the proteasome and autophagy, and reduced oxidative stress, opening new avenues for the exploitation of (poly)phenols in nutrition and health.

Introduction

(Poly)phenols are natural compounds found in a wide variety of foods which either as dietary or nutraceutical supplements, are known for their health benefits [1]. Epidemiological and clinical studies showed that (poly)phenols can reduce the incidence and prevalence of cardiovascular diseases, cancer, diabetes, inflammation and age-related disorders, as pure (poly)phenols, in extracts or through diet [1-6]. The term polyphenol is used to describe a structure with at least one aromatic ring with one or more hydroxyl groups attached [6]. However, compounds with only one phenolic ring are also referred to as polyphenols, as hydroxycinnamates and phenolic acids. Thus, recently polyphenols have been referred to as (poly)phenols [6]. However, for the rational recommendation of (poly)phenols in nutrition their bioaccessibility and the mechanism of action must be dissected. Thus, the understanding of the digestion influence on the bioactivities of dietary (poly)phenols is imperative. Studies of digestion in humans and in animal models pose ethical, time and cost concerns. Thus, *in vitro* digestion models to predict phytochemical changes during digestion offer several advantages, including the suitability for mechanistic studies [7]. Moreover, this strategy constitutes a high-throughput approach suitable for mechanistic studies of dietary (poly)phenols in cellular models of disease [7].

Several studies support the beneficial effects of (poly)phenols. Namely, animal studies showed that dietary (poly)phenol metabolites are bioavailable with accumulation in the brain after consumption [8, 9], and can reverse and prevent age-related cognitive decline and neurodegeneration [10]. Thus, (poly)phenols are attractive as they can easily be incorporated as dietary supplements.

PD is an age-related neurodegenerative disease that has currently no effective therapy [11]. The misfolding and aggregation of alpha-synuclein (aSyn) is deeply associated with PD pathogenesis [12], but the precise underlying molecular mechanisms remain elusive. Among these, oxidative stress and mitochondrial dysfunction are common factors, contributing to disturbances in cellular processes that result in irreversible cell damage and death [13]. In particular, the excessive accumulation of unfolded proteins and oxidative stress

that are typical in PD, result in the failure of the endoplasmatic reticulum (ER) to cope with the excess of protein load, a process denominated by ER stress [14, 15].

The ER has several important functions in the cell as it is responsible for the synthesis of one-third of the total proteome, and is involved in the folding, maturation and post-translational modification of proteins [14]. In response to ER stress cells activate the unfolded protein response (UPR) [14-16], leading to the transcription of genes related with protein homeostasis, including chaperones, and protein degradation and secretion pathways [14]. Sustained ER stress and UPR activation may overwhelm cellular protective mechanisms, ultimately triggering apoptosis [14]. The ER-associated protein degradation (ERAD) is a cellular pathway which targets misfolded proteins for ubiquitination and subsequent degradation by the ubiquitin–proteasome system (UPS) [16]. Macroautophagy, hereafter designated as autophagy, is also initiated in response to ER stress caused by misfolded proteins, via ER-activated autophagy (ERAA) [16, 17], which induces a partial UPR and a calcium-mediated signalling cascade. ERAA is a degradation pathway and serves the function of mitigating ER stress and suppressing cell death, as ERAD [16].

The clearance of soluble aSyn can occur both via the UPS and chaperone-mediated autophagy (CMA) [18]. Under pathological conditions, aSyn inhibits the proteasome and impairs CMA [19], leading to the upregulation of autophagy [20]. On the other hand, the degradation of aggregated aSyn occurs by autophagy [21, 22]. Nonetheless, the relationship between autophagy and disease is unclear.

We previously showed that (poly)phenols can protect against aSyn toxicity by promoting its clearance trough autophagy and by modulating aSyn fibrillization [23]. (Poly)phenols from *A. unedo*, a native species in regions of Mediterranean climate, were shown to hold potential for the treatment of hypertension [2], cardiovascular diseases [24] and cancer [3, 25]. Although, its potential for neurodegenerative disease has not been explored. Here, a pioneer nutritional approach was taken to study the bioactivities of (poly)phenol-digested metabolites (PDM), obtained after *in vitro* gastro-intestinal digestion [26] of *A. unedo* (edible leaves and fruits). The chemical alterations occurring during digestion were analyzed by LC-MS and the molecular mechanisms of PDM protection via regulation of autophagy and other targets of aSyn toxicity were analyzed. Interestingly, PDM from *A. unedo* leaves

displayed a significant protection against aSyn and H_2O_2 toxicity in yeast and human H4 cells, respectively. In addition, they reduced aSyn aggregation and induced its clearance trough the activation of autophagy and proteasome function. These metabolites were also able to reduce oxidative stress and mitochondria impairment induced by aSyn. We also found that PDM regulated transcription of genes involved in the unfolded protein response and Sir2 pathways. In total, our pioneering study of the effects of PDM in PD models revealed protective molecular mechanisms, with emphasis on the clearance of misfolded proteins and modulation of autophagy. Altogether, our study opens novel avenues for the exploitation of nutraceuticals/therapeutics based on (poly)phenols for neurodegenerative diseases.

Results

(Poly)phenol-digested metabolites from *A. unedo* leaves protect against aSyn-induced toxicity

We used an established model of PD based on the expression of human aSyn in yeast cells [19, 27] to dissect the mechanisms of protection of *A. unedo* PDM. The concentration of 62 µg GAE.mL^{-1} was selected as non-toxic based on viability assays (Fig. S1). Yeast cells grown in raffinose medium were transferred to galactose medium, to induce aSyn expression, and treated at the same time with PDM for 6 h. After this treatment, cellular viability was assessed by spotting assays (Fig. 1A). Importantly, we observed that leaf or fruit PDM, had no effect on the control cells growth. Remarkably, these PDM were able to suppress toxicity induced by aSyn expression (Fig. 1A). Protection also observed by the reduction of PI positive cells assessed by flow-cytometry (Fig. S2).

aSyn toxicity in yeast cells is associated with inclusion formation [19]. We then assessed if the enhancement in cell viability was accompanied by alterations in the inclusion formation of aSyn, by fluorescence microscopy. Interestingly, treatment with leaf PDM significantly reduced the percentage of cells displaying aSyn inclusions (Fig. 1B). However, the treatment with fruit PDM had no effect on inclusions formation (Fig. 1B).

Oxidative stress is another central event in PD being a known trigger of aSyn misfolding and aggregation. Conversely, aSyn aggregation induces mitochondrial dysfunction and ROS production [28]. Accordingly, we then analyzed the effect of PDM treatment on ROS levels in yeast cells expressing aSyn. ROS levels were determined by flow cytometry (FCM), using 2',7'-dichlorfluorescein-diacetate (DCFHDA) (Fig. 1C, left panel), a dye that reacts with ROS and acts as an indicator of the general oxidative state of the cell. We observed that PDM had no significant effect on the levels of ROS, although an increase in ROS was observed in cells expressing aSyn (Fig. 1C) as previously described [28]. Superoxide radical levels were also evaluated by FCM, using dihydroethidium (DHE). Superoxide radical is an indicator of mitochondrial metabolic function [29], a known target of aSyn-induced toxicity [30].

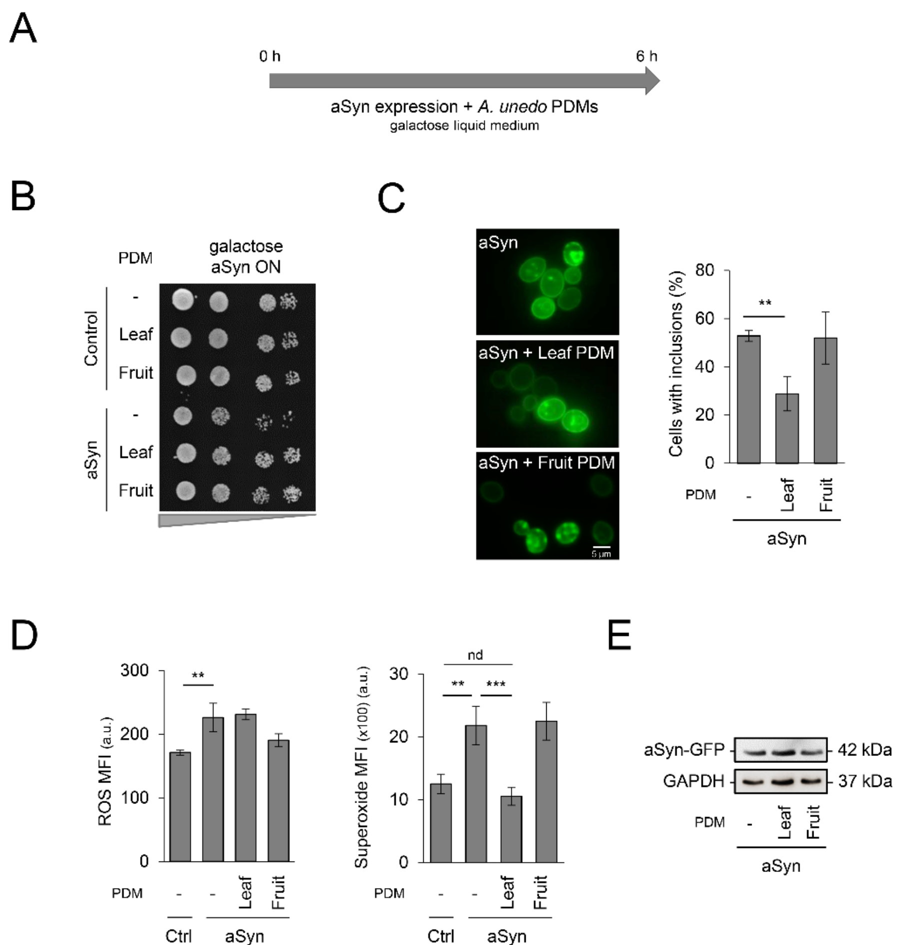

FIGURE 1. *A. unedo* leaf PDM reduces aSyn-induced toxicity, inclusions formation and superoxide radical levels. A) Yeast cells transformed with the aSyn encoding plasmid or the empty vector (control, ctrl), were treated with 62 µg GAE.mL^{-1} of leaf or fruit PDMs for 6 h, in galactose liquid medium. **B)** Cell viability assessed by spot assays, after PDMs treatment in galactose liquid medium. **C)** Fluorescence microscopy images of yeast cells (left panel) and respective percentage of cells with aSyn inclusions (right panel). **D)** ROS levels evaluated by DCF median fluorescence intensity (MFI) (left panel) and superoxide radical levels evaluated by DHE MFI (right panel), assessed by flow cytometry. Results are represented in arbitrary units (a. u.) **E)** aSyn expression levels assessed by western blot, GAPDH was used as loading control. A representative results is shown and values represent the mean ± SD of three independent experiments. Statistically significant differences between the indicated treatments are shown, **p<0.01, ***p<0.001 or nd as not different for p<0.05.

As observed before, aSyn expression led to an increase in superoxide production (Fig. 1C, right panel) [23]. Treatment with leaf PDM substantially reduced the levels of superoxide in cells expressing aSyn (Fig. 1C, right panel).
We next assessed whether the differences in toxicity and inclusion formation were due to different expression levels of aSyn induced by the PDM treatments. Using western blot analyses, we found that the PDM did not affect aSyn protein expression levels (Fig. 1D).

Overall, these results suggest that leaf PDM are cytoprotective in a yeast model of PD by reducing inclusion formation and improving redox homeostasis. For this reason, we continued our study focusing on leaf PDM, leading us to do the chemical profile of its (poly)phenols.

Chemical characterization of leaf fractions

LC-MS analysis of the *A. unedo* leaf extracts before (original) and after (PDM) digestion (Table 1) confirmed that they were mainly composed of (poly)phenols previously described as having bioactivities [2, 6, 8, 23]. The profiles before and after digestion were similar, with the same main compounds being identified, suggesting substantial stability to digestion (Table 1). The major flavonols present were quercetin hexoside, quercetin pentoside, myricetin-3-rhamnoside, and kaempferol glucosides. As noted in previous *in vitro* digestion experiments this class of (poly)phenols is described as being stable to digestion [26]. The galloylated derivatives and epicatechin were less stable and their levels were reduced in the PDM. Additionally, three, as yet, unknown compounds arose after digestion (Table 1).

PDM modulate the oligomerization of aSyn

In order to determine the effect of leaf PDM on the biochemical nature of the aSyn species formed in yeast cells, we performed centrifugation in sucrose gradients, as previously described [31]. aSyn oligomeric species were found dispersed throughout the various sucrose gradient fractions (Fig. 2A). Interestingly, upon treatment with PDM, aSyn species were predominantly present in fractions corresponding to smaller molecular weight species (Fig. 2A, right panel). We confirmed that these changes were not due to differences in the loading of the total protein (Fig. 2B). From these results we concluded that leaf PDM

modulates the nature of aSyn species formed in yeast cells, reducing the formation of higher molecular weight oligomeric species of aSyn.

Table 1: (Poly)phenol metabolites present in *A. unedo* leaf before (original) and after *in vitro* digestion (PDM). (Poly)phenol putative identifications, structure and classes are presented, as well as MS (M/Z and MS^2) and LC (retention time and PDA) data.

	Class	Compound	MS Data		LC Data		Original	PDM
			M/Z	MS^2	Retention Time (min)	PDA (nm)		
Flavonoids					[a]		[a]	
	Flavanols	Epicatechin (formate adduct	**289** (335)	**245,** 205,179	3.48	280	+	-
	Flavonols	Quercetin hexoside	463	**301**	8.30	350	+	+
		Quercetin pentoside	433	**301**	8.99	350	+	+
		Myricetin-galloyl –hexoside	631	**479, 301**	5.87	280, 350	+	-
		Myricetin-3-rhamnoside	463	**317**	8.09	350	+	+
		Kaempferol hexoside	447	**285**	9.24	350	+	+
		Kaempferol pentoside	417	**285**	9.75-9.93	280, 350	+	+
		Kaempferol glucoside	431	**285**	10.01	280, 350	+	+
	Ellagitannin	Strictinin	633	**463**, 301	5.22	280, 350	+	-
	Phenolic acid	Digalloyl shikimic acid	477	**325**	4.71	280	+	-
	Unknowns	Unknown a (261-hexoside)	423	**261**	3.74	280	+	+
		Unknown b	431	179, 161	5.23	280	-	+
		Unknown c	405	**359**, 181, 161	5.54	280, 350	-	+
		Unknown d	391	281, 137	7.25	280, 350	-	+

[a]The main metabolites were identified using PDA maxima, *m/z* and MS^2 fragmentation data. PDA detector range was 200-600 nm and mass spectrometer mass range was *m/z* 80-2000 [32].

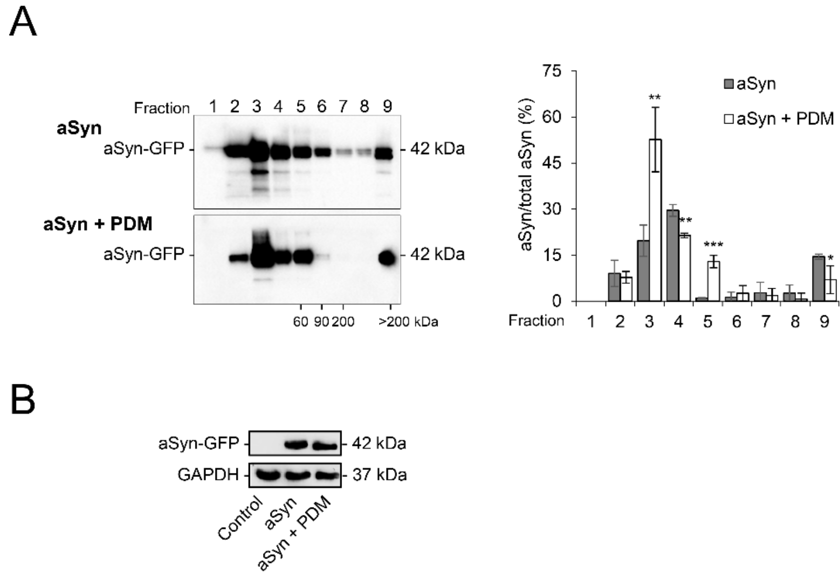

FIGURE 2. *A. unedo* leaf PDM modulates the nature of aSyn oligomeric species. Yeast cells transformed with the aSyn encoding plasmid were treated with 62 μg GAE.mL^{-1} of leaf PDM for 6 h, in galactose medium. **A)** Oligomeric aSyn species resolved using sucrose gradients. The resulting fractions were separated on an SDS-PAGE gel followed by immunoblotting for aSyn (left panel); quantification of aSyn in each fraction normalized by total aSyn (right panel). **B)** The samples submitted to the sucrose gradients were evaluated for aSyn expression levels assessed by western blot. Significantly different results are indicated between "aSyn" and "aSyn + PDM" treatment for each fraction. A representative result is shown and values represent the mean ± SD of three independent experiments. Statistically significant differences between the indicated treatments are shown, *p<0.05, **p<0.01, ***p<0.001.

PDM promote the clearance of aSyn by restoring proteasomal and autophagic activities

The impairment of protein clearance pathways is implicated in the pathogenesis of PD, leading to the accumulation of misfolded and aggregated aSyn [12, 33]. Thus, we next evaluated the effect of PDM treatment on aSyn clearance (Fig. 3A). Briefly, 6 h after induction of aSyn expression in galactose medium in the presence of PDM (0 h of clearance), cells were switched to glucose-containing medium in order to repress aSyn expression. Cells were then grown in glucose medium for 18 h (18 h clearance) and the degradation/clearance of aSyn was assessed by western blotting. Cells treated with PDM

during the induction period presented lower levels of aSyn after 18 h of clearance (Fig. 3A), suggesting that (poly)phenols promoted the degradation of aSyn. Thus, we then analyzed the effect of PDM on proteostasis pathways.

The clearance of aSyn has been associated with both UPS and autophagy-lysosome pathway [18]. The effect of PDM on proteasome activity was analyzed using an unstable GFP reporter (GFPu) consisting of GFP fused with a constitutive degradation signal (CL-1) that promotes its rapid degradation by the UPS [19]. FCM analysis revealed that, upon aSyn expression, the levels of GFPu considerably increased (Fig. 3B) indicating that aSyn impaired proteasome function, as previously reported [19, 23]. Interestingly, treatment with PDM reduced the levels of GFPu in the cells expressing aSyn, indicating that the PDM attenuated proteasome impairment (Fig. 3B). Treatment with PDM had no effect on the levels of GFPu in control cells not expressing aSyn (Fig. 3B).

Subsequently, we tested whether autophagy was affected by PDM using the GFP-Atg8 processing assay as a reporter [34, 35]. Atg8 is one of the key molecules involved in autophagy and its conjugation to the autophagosomal membrane, through an ubiquitin-like conjugation system, is essential for autophagy in eukaryotes [35]. We observed that aSyn expression lead to a significant increase in Atg8 induction and reduced autophagic flux (Fig. 3C), suggesting that aSyn interfered in autophagy function as previously described [23]. Remarkably, when aSyn-expressing cells were treated with PDM the autophagic flux was restored to control levels (Fig. 3C). However, in the control cells not expressing aSyn, both Atg8 induction and autophagic flux remained unchanged when cells were treated with PDM (Fig. 3C).

To reinforce the above observations we then investigated the proteasome and autophagy pathways, by evaluating the mRNA levels of *RPN4* and *ATG8* genes since their transcriptional regulation is well characterized. *RPN4* encodes for a transcription factor that regulates the expression of proteasome genes [36], and is upregulated by the UPR [37]. We observed that *RPN4* transcript levels increased upon aSyn expression (Fig. 3D). In contrast, in cells treated with PDM the *RPN4* transcript levels did not increase (Fig. 3D). Similarly, aSyn expression induced *ATG8* expression, as expected, considering the Atg8 induction

observed in the GFP-Atg8 processing assay (Fig. 3C). However, upon PDM treatment, *ATG8* transcript levels were similar to those in control cells not expressing aSyn (Fig. 3D).

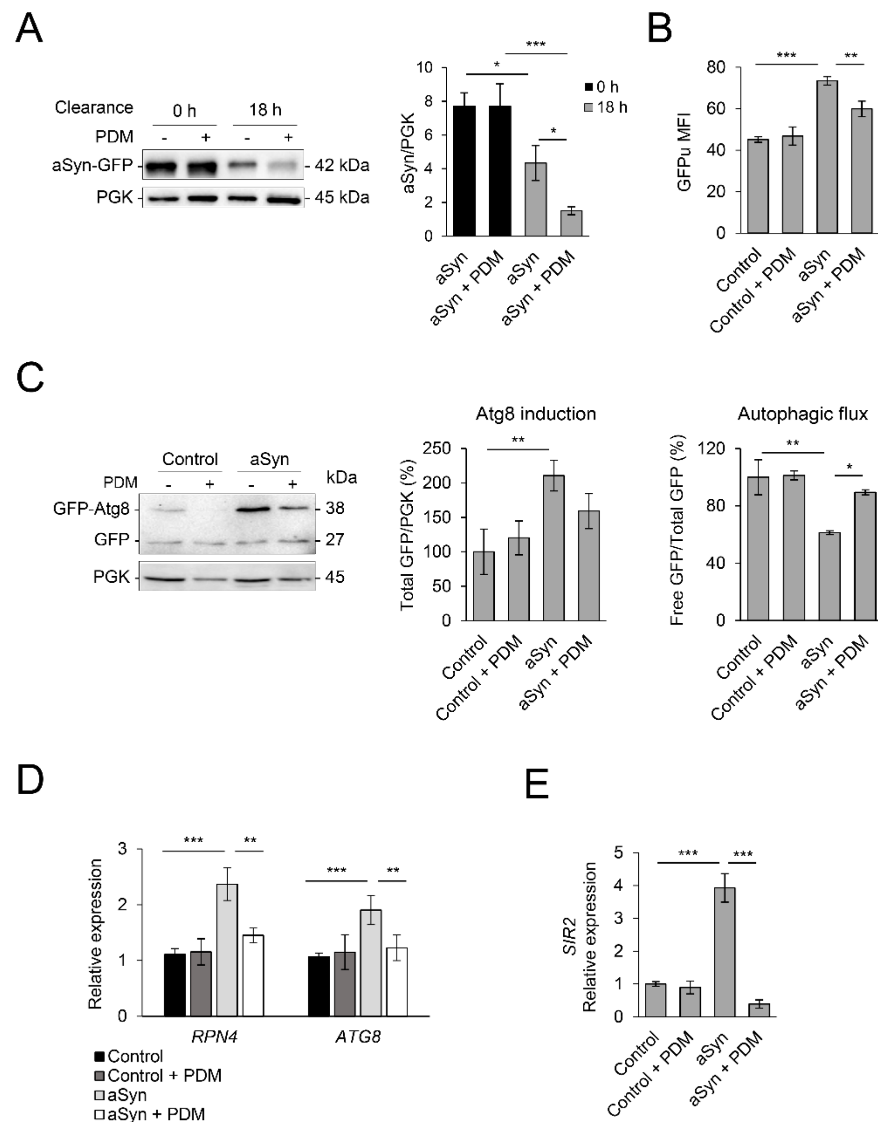

FIGURE 3. PDM increases the clearance of aSyn by promoting autophagy and proteasomal function. Yeast cells transformed with the aSyn encoding plasmid or the empty vector (control), were treated with 62 μg GAE.mL^{-1} of leaf PDM for 6 h, in galactose medium. **A)** Clearance of aSyn evaluated by western blot at 6 h of aSyn induction (0 h clearance) and after18 h in glucose medium (18 h of clearance) (left panel) and respective quantification normalized to PGK levels. **B)** Proteasome impairment evaluated by flow cytometry (FCM) using GFPu median fluorescence intensity (MFI), after

6 h of aSyn induction. **C)** Autophagy evaluated by GFP-Atg8 processing assay assessed by western blot (left panel), after 6 h of aSyn induction. Atg8 induction quantified by the total GFP signal (GFP-Atg8 and free GFP signal, detected with anti-GFP) (middle panel); autophagic flux quantified by measuring the vacuolar degradation of the Atg8 domain reporter (ratio of free GFP to total GFP signal) (right panel). **D)** *RPN4* and *ATG8* gene relative expression levels assessed by qRT-PCR. **E)** *SIR2* relative expression levels assessed by qRT-PCR. A representative results is shown and values represent the mean ± SD of at least three independent experiments. Statistically significant differences between the indicated treatments are shown, *p<0.05, **p<0.01, ***p<0.001.

To gain further insight into the regulation of proteostasis by PDM treatment, we then analyzed a known inducer of autophagy and modulator of aSyn toxicity, the Sir2p [38]. Sir2 is a deacetylase of the sirtuin family and functions as a regulator of autophagy and mitophagy [39]. Upregulation of Sir2 is linked with the induction of excessive autophagy and a resulting increase in aSyn toxicity, in aged yeast cells [38]. Consistently, we observed that aSyn expression increased the transcript levels of *SIR2* under the conditions tested (Fig. 3E). However, upon treatment with PDM, the *SIR2* expression levels were similar to the control levels (Fig. 3E). Altogether, these results indicate that the PDM studied here might play an important role in regulating autophagy, and that one of the mechanisms involved might be through Sir2 modulation.

PDM modulate the unfolded protein response and mitochondrial function

Due to the observed promotion of aSyn clearance by PDM, we decided to evaluate the effect of treatment on the expression levels of a set of UPR related genes. Thus, we assessed the mRNA levels of *KAR2*, *LHS1, HSP26* and *HRD1*. Here, we observed that aSyn expression induced an increase in the expression levels of all the UPR genes evaluated (Fig. 4A). On the other hand, PDM treatment reduced the levels of *LHS1*, *HRD1* and *HSP26* transcripts (Fig. 4A), suggesting that PDM were able to attenuate the UPR and the associated ER stress.

Oxidative stress and mitochondrial dysfunction are known triggers of protein misfolding and ultimately result in ER stress. *SOD1*, *SOD2* and *HAP4* mRNA levels were analysed to gain additional insight into the effect of PDM treatment on mitochondrial function (Fig. 4B). aSyn expressing cells displayed lower levels of *SOD1* and *SOD2* and, remarkably, higher levels of *HAP4*, in comparison to the control cells (Fig. 4B). Notably, upon treatment

with PDM, *SOD1* and *SOD2* and *HAP4* levels were similar to the control cells. For *HAP4* its levels in aSyn expressing cells of were drastically reduced by PDM (Fig. 4B).

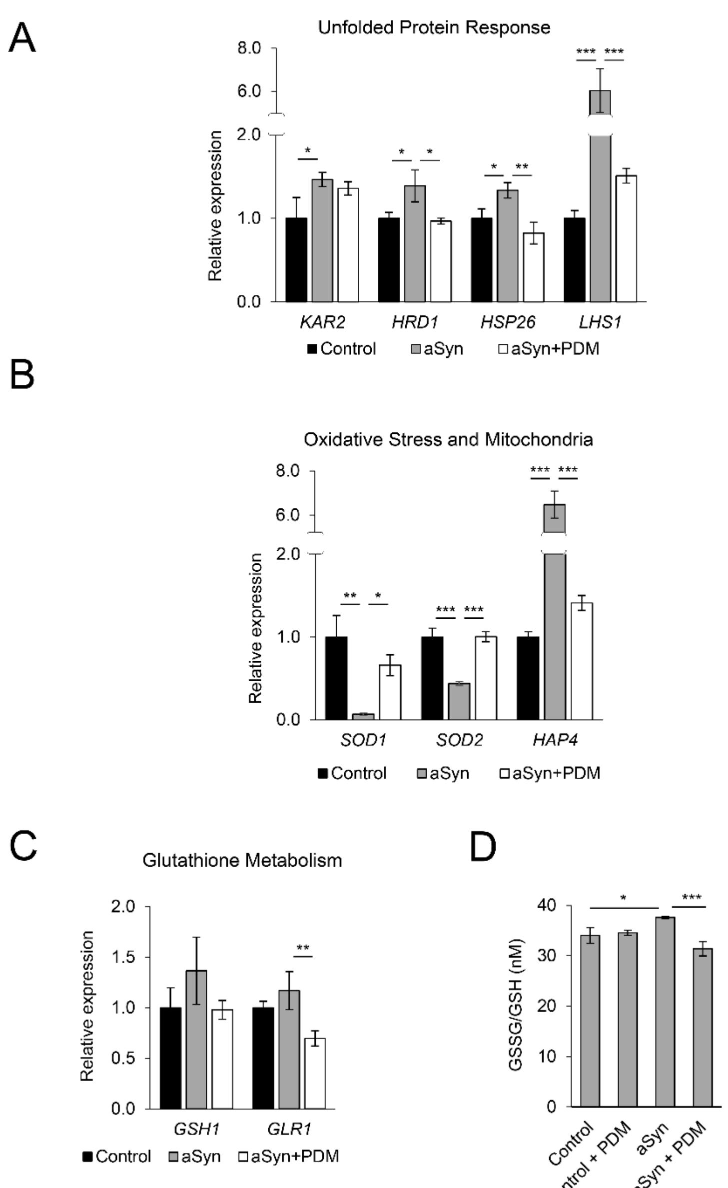

FIGURE 4. Effect of PDMs in the unfolded protein response and oxidative stress. Yeast cells were treated with 62 μg GAE.mL^{-1} of leaf PDM for 6 h, in galactose medium. **A)** Transcriptomic analyzes of genes involved in the unfolded protein response, assessed by qRT-PCR. **B)** Transcriptomic analyzes of genes involved in oxidative stress response (*SOD1*, *SOD2*) and mitochondrial biogenesis (*HAP4*), assessed by qRT-PCR. **C)** Transcriptomic analyzes of genes involved in glutathione

metabolism, assessed by qRT-PCR. **D)** GSH/GSSG levels assessed by HPLC. A representative result is shown and values represent the mean ± SD of three independent experiments. Statistically significant differences between the indicated treatments are shown, *p<0.05, **p<0.01, ***p<0.001.

We then evaluated the major low-molecular-weight redox buffer, glutathione (GSH), a tripeptide that plays a pivotal role in the protection against oxidative damage, detoxification from xenobiotics, and endogenous toxic metabolites [40]. We assessed both the levels of the key enzymes involved in GSH synthesis and also the ratio of oxidized glutathione to reduced glutathione (GSSG/GSH). Interestingly, the levels of *GSH1* and *GLR1* were not significantly affected by aSyn expression. However, a slight but significant reduction in *GLR1* mRNA levels was observed in cells expressing aSyn treated with PDM (Fig. 4C). The cellular glutathione pools were also determined by HPLC, since the GSSG/GSH ratio is a measure of the cell's redox state [40]. We observed that aSyn expression increased the GSSG/GSH ratio, suggesting an increase in oxidative stress. Treatment with PDM reduced this ratio, suggesting the reduction of oxidative stress.

PDM modulate aSyn aggregation, protect from oxidative stress, and promote mitochondrial function in human cells

Next, we investigated the effect of the selected PDM in a human cell model of aSyn aggregation where we can induce the formation of LB-like inclusions (Fig. 5A, left panel) based on the co-expression of a C-terminally modified version of aSyn (SynT) and Synphilin-1, a protein that enhances aSyn aggregation [41, 42]. We co-transfected cells with plasmids encoding for SynT and Synphilin-1 and, 24 h after, we treated cells with PDM for 16 h. We observed that PDM treatment, increased the percentage of cells without aSyn inclusions and reduced the percentage of cells presenting more than 10 inclusions (Fig. 5A, right panel). Importantly, the effect observed was not due to differences in SynT levels due to PDM treatment (Fig. 5B).

The PDM protective capacity against oxidative injury was also analyzed in human H4 cells treated with H_2O_2 (Fig. 5C). We observed that the pre-treatment with PDM for 16 h, prior to stress with 600 µM H_2O_2 for 6 h, protected cells from oxidative stress-induced cell death, as observed by the reduction of the number of PI positive cells (Fig. 5C). These data

show that the PDM protect human cells from oxidative stress, validating the results obtained in yeast.

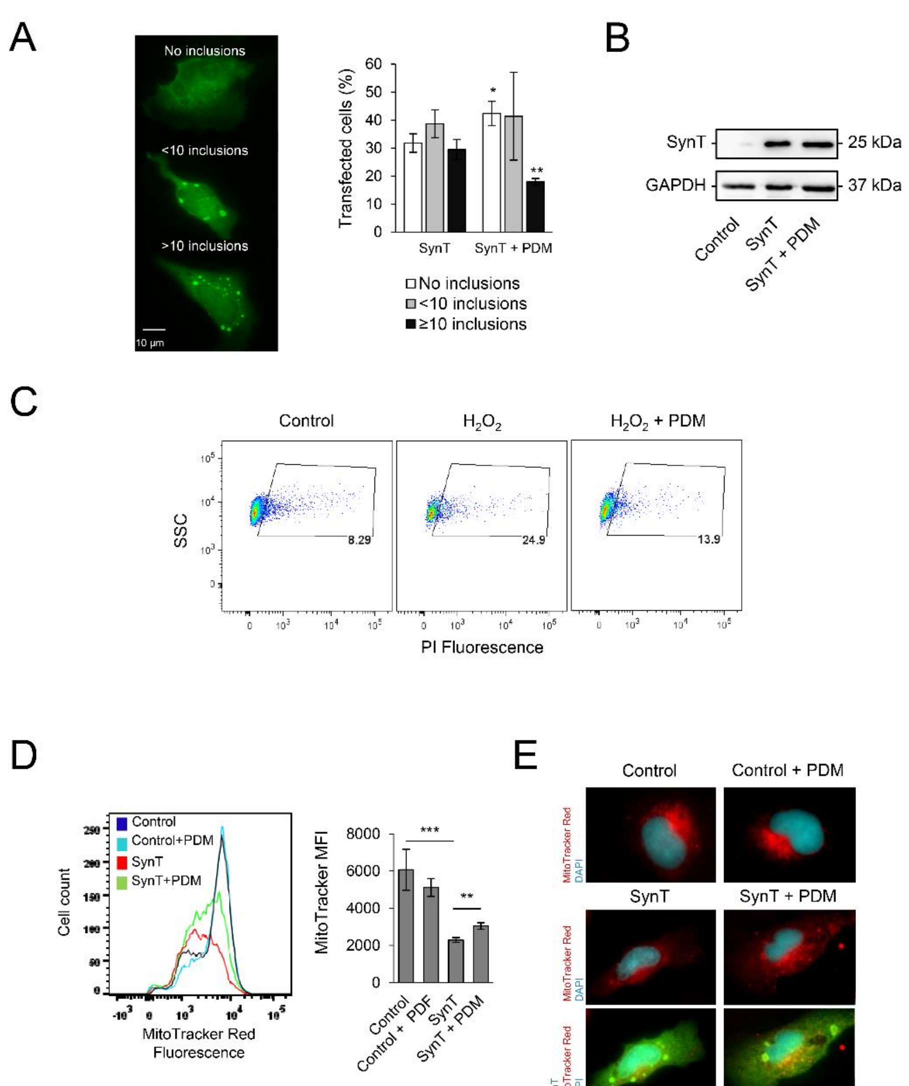

FIGURE 5. PDMs modify the distribution of aSyn inclusions, protect from H_2O_2-induced toxicity and promote mitochondrial function in H4 cells. A) H4 cells were transfected with SynT (SynT and Synphilin-1) for 24 h and treated with 2 µg GAE.mL^{-1} leaf PDM for 6 h. Subsequently, cells were subjected to immunofluorescence labeling and the percentage of cells with inclusions was determined by fluorescence microscopy (left panel). Cells were classified by cells without inclusions (□), with less than 10 inclusions (■), or with 10 or more inclusions (■) (right panel). **B)** SynT levels of H4 cells transfected with the empty vector (control), co-transfected with SynT and Synphilin-1 (SynT) or

treated with leaf PDM (SynT + PDM), assessed by western blot. GAPDH was used as loading control. **C)** H4 mammalian cells were pre-treated with leaf PDM for 16 h, then subjected to 600 μM of H_2O_2 for 6 h. Viability was assessed by PI fluorescence *versus* side scatter (SSC) (left panel), determined by flow cytometry. **D)** Mitochondrial membrane potential determined using MitoTracker Red by flow cytometry; histogram of MitoTracker Red fluorescence (left panel) and respective median fluorescence intensity (MFI) (right panel) of H4 cells transfected with the empty vector (control), co-transfected with SynT and Synphilin-1 not treated (SynT) or treated with leaf PDM (SynT + PDM). **E)** H4 cells transfected with the empty vector (control), co-transfected with SynT and Synphilin-1 (SynT) or treated with leaf PDM (SynT + PDM) were immunostained for SynT (green).Mitochondria were visualized using MitoTracker Red (red) while Nuclei were stained with DAPI (blue). A representative result is shown and values represent the mean ± SD of at least three independent experiments. Statistically significant differences between the indicated treatments are shown, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

The mitochondria work as generators and targets of ROS [13] and its function is impaired by aSyn [29, 30]. Mitochondrial function was studied using MitoTracker Red, a dye that stains mitochondria in live cells and whose accumulation is dependent on mitochondrial membrane potential, a parameter linked to mitochondrial function. In cells expressing SynT, MitoTracker Red fluorescence was lower than in control cells, indicating reduced mitochondrial membrane potential and suggesting impairment of mitochondrial function. On the other hand, treatment with PDM of cells expressing SynT increased the MitoTracker Red signal, suggesting a rescue of the mitochondrial function (Fig. 5D). Mitochondria were also evaluated by fluorescence microscopy (Fig. 5E), confirming the FCM data (Fig. 5E).

Discussion

PD is currently an incurable disorder affecting a growing number of people due to the aging of the population [11]. Therefore, it is urgent to understand the molecular mechanisms underlying PD pathogenesis and to identify novel therapeutic targets and strategies. Phenolic compounds, in food, pure, or in extracts, are of great interest in nutrition and medicine. They have been associated with the prevention of cancer, chronic and neurodegenerative diseases [1-5]. Thus, (poly)phenols are attractive compounds in the context of neurodegenerative diseases, since public health policies are focused on the prevention of dementia, through the promotion of a healthy life style, natural nutrition and supplementation [1].

In this pioneer study of digested metabolites in PD, we found that PDM from *A. unedo* leaves offered a significant cytoprotection in established yeast and human cell models of synucleinopathies [19, 27, 41, 42]. *A. unedo* possess several useful biological properties [2, 24, 25, 43], nevertheless it neuroprotective potential was not explored. After digestion the obtained PDM was manly composed by myricetin-3-rhamnoside, quercetin-hexoside and kaempferol glucosides. The last has been described as being neuroprotectors against ischemic brain injury and neuroinflammation, in rats [44]. While, myricetin and quercetin were described to efficiently inhibit aSyn fibrillization [45].

Interestingly, in a previous study we observed that the original fraction from *A. unedo* leaves is not protective against aSyn-induced toxicity [23]. Therefore, the results here obtained indicate that the digestion potentiates the beneficial effects of (poly)phenols in *A. unedo*, reinforcing their nutritional relevance. Importantly, digestion was also found to potentiate neuroprotective proprieties of blackberry (poly)phenols[46].

PD pathogenesis is associated with proteostasis dysfunction and inefficient protein clearance [12, 20]. Autophagy was shown to be required for the degradation of aSyn aggregates under pathological conditions [20, 23, 29, 31, 33]. Nevertheless, recent studies reported that increased autophagy/mitophagy, promoted by aSyn, has a deleterious effect in aged cells [29, 38]. This appears counterintuitive, as autophagy is generally considered to be a pro-survival process in protein misfolding diseases [20]. However, autophagy is a complex pathway and,

depending on the conditions and models used, it can be either a survival or death mechanism. In the conditions tested herein, aSyn induces the activation of autophagy, as measured by Atg8 protein and mRNA levels. However, this autophagy is dysfunctional, as observed by the reduced autophagic flux. Ultimately, the excessive activation of a dysfunctional autophagy will lead to a loss of selectivity, resulting in the trapping of functional competent organelles in autophagosomes, contributing to the toxicity observed. In fact, PDM reduced aSyn toxicity and aggregation by promoting a functional autophagy, as observed by the increased autophagic flux. Altogether, our findings provide a reasonable explanation for aSyn toxicity as being mediated by autophagy. Thus, the beneficial or detrimental role of autophagy on aSyn toxicity must be studied in the context of its functionality and selectivity. In agreement, our previous study showed that (poly)phenols preferentially affect the autophagic function [23], which is impaired by aSyn expression.

To elucidate the pathways/molecules associated with aSyn-induced autophagy activation, we investigated the UPR pathway and the levels of ROS. ERAD and ERAA, related with the UPS and autophagy, are both mitigators of ER stress [16, 17, 37]. Accordingly, increasing evidence suggests the existence of a complex cross-talk between different proteostasis pathways, processes known to play distinct roles in the clearance of specific species of aSyn [18, 21, 22, 31].

Here we analysed the expression of ER stress associated genes, where Kar2 and Lhs1 are HSP70 molecular chaperones found in the ER, where they mediate protein folding [47, 48]. Kar2 negatively regulates the UPR via interaction with Ire1 [14, 47], a major regulator of the UPR . Lhs1 acts as a nucleotide exchange factor for Kar2 and is upregulated by the UPR [48]. Hsp26 is a cytosolic chaperone that binds and prevents unfolded proteins from irreversibly forming large protein aggregates [49]. Hsp26 activity is found only under stress conditions, where it is strongly induced [49]. Hrd1 is an ubiquitin ligase responsible for recognizing and ubiquitinating misfolded proteins in the ER, for further degradation by the proteasome (ERAD) and is induced in UPR conditions [50] (Fig. 6).

Our findings demonstrate that aSyn-elicited ER stress and the concomitant activation of the UPR, might underlie the observed induction of genes related with autophagy and proteasomal function. PDM reduced both autophagy activation and the induction of UPR

genes, while promoting autophagy and proteasome function (Fig. 6). In line with this observation, it is known that Rpn4, a transcription factor that stimulates the expression of proteasome genes, is regulated by the 26S proteasome in a negative feedback control mechanism [36].

The reduction in the levels of *LHS1* and *RPN4* mRNA by PDM is in agreement with an extensive screen for small molecules capable of attenuating aSyn toxicity [51]. In this study, aSyn expression induced the upregulation of *LHS1* and *RPN4*, among other genes, and the protective compounds markedly reversed the transcriptional changes induced by high levels of aSyn [51], as observed here for the PDM under study.

ROS, particularly superoxide anions, are crucial signaling molecules implicated in the control and regulation of autophagy and aging [52]. Sod1 and Sod2 are superoxide dismutases responsible for detoxifying superoxide radical, which are thinly regulated at transcription level in response to oxidative stress [53]. Sod1 is mainly cytosolic while Sod2 is found in the mitochondria, both are strongly implicated in the aging of yeast cells, and their deletion dramatically reduces the chronological and replicative life span of yeast [52]. Moreover, the efficiency of the protein folding status of the ER depends on specific redox condition in its lumen [14]. Our data suggest that the upregulation of *SOD1* and *SOD2* might be underlying mechanism behind PDM-mediated decreased in superoxide anion levels, a ROS involved in mitochondrial functionality [13]. These observations are further substantiated by recent studies of the activity of antioxidant enzymes in PD models. Induction of PD by the expression of mutant aSyn [54] or DJ-1 [55] in flies, and MPP+ exposure in a human cell line [56], resulted in reduced SOD activity and increased production of superoxide. It was proposed that the increase in ROS levels observed in the PD models was due to a deficient activity of the antioxidant enzymes. In addition, treatment with protective compounds increased SOD activity and rescued the PD phenotype [54-56]. Moreover, treatment with (poly)phenols reduced the ratio of oxidized/reduced glutathione (GSSG/GSH), supporting their role against oxidative stress damage. In our study, the evidence that PDM improved oxidative homeostasis and mitochondrial function was further confirmed in human cells, as PDM increased mitochondrial membrane potential in cells expressing aSyn.

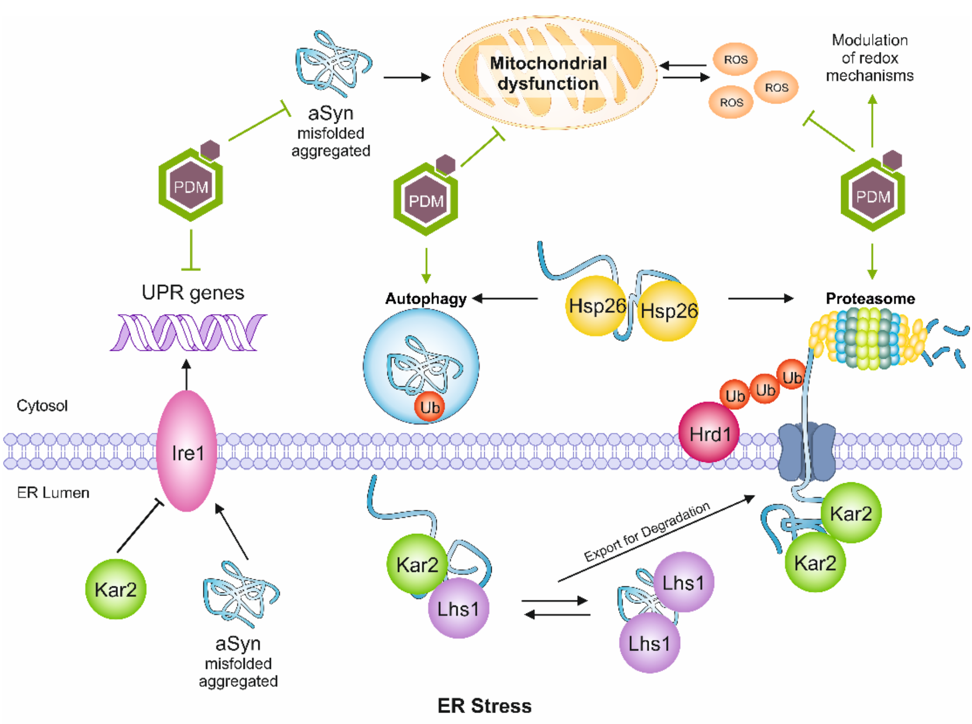

FIGURE 6. Schematic representation of the mechanism of protection by *A. unedo* leaf PDM. Accumulation of unfolded proteins (such as aSyn) or oxidative stress leads to endoplasmatic reticulum (ER) stress with activation of the unfolded protein response (UPR), leading to oligomerization of the kinase Ire1, and transcription of genes related with protein-folding homeostasis. Kar2 is a negative regulator of UPR via interaction with Ire1. When unfolded proteins accumulate Kar2 is displaced from Ire1, to act as a chaperone aiding proteins conformation. Direct activation of Ire1 by unfolded proteins is an alternative mode for UPR induction. Lhs1, an ER chaperone, mediates protein folding and interacts with Kar2. Cytosolic chaperones also assist aSyn conformation, as Hsp26. Proteins failing to be correctly folded are exported from the ER, a process involving Kar2. Hrd1 is an ubiquitin ligase responsible for recognizing and ubiquitinating misfolded proteins. Afterward ubiquitination, proteins are either degraded in the cytosol by the proteasome or by autophagy, pathways that are both upregulated by the UPR. Our findings showed that aggregated aSyn induced reactive oxygen species (ROS) production and elicited ER stress, with concomitant induction of UPR gene expression. PDM reduced aSyn aggregation and the induction of UPR, while promoting autophagy and proteasome function, pathways involved in aSyn degradation. Moreover, PDM reduced oxidative stress and mitochondrial dysfunction, by modulation of redox homeostasis. Overall, PDM increased cell viability by a pleiotropy protective role.

Mammalian SIRT1 was recently described as an inducer of autophagy under starvation conditions, and is required to sustain autophagy through deacetylation of autophagic regulators, including Atg5, Atg7, Atg8 and Atg12 [57]. Here, we also investigated the effect of PDM on Sir2, the yeast ortholog of SIRT1. Notably, *SIR2* overexpression increases aSyn toxicity by regulating *ATG8* and, particularly, *ATG32* mRNA, leading to excessive mitophagy [38]. Our findings revealed that aSyn expression induced *SIR2* and, impressively, PDM treatment maintained *SIR2* expression at basal levels, in line with the autophagy induction results, and the described effect of acetylation on autophagy regulation [57].

Altogether, the effect of PDM on *SIR2* expression, autophagy flux and aSyn toxicity protection are in agreement with previous studies where it was observed that deletion of the *SIR2* gene alleviated aSyn toxicity and drastically inhibited autophagy induction [38].

Overall, PDM reduced aSyn toxicity and aggregation, enhanced the efficiency of the ERAD and ERAA by the promoting the proteasome and autophagy function, and reduced oxidative stress. Thus, these compounds constitute a promising source for nutraceutical/therapeutic strategies against PD and other synucleinopathies. Ultimately, our study opens novel opportunities for the exploitation of (poly)phenols in nutrition and health.

Materials and methods

Plant material and extraction procedure

Fruits and leaves of *A. unedo* L. were collected by random sampling in an extensive area of Arrábida Natural Park. Plant material was harvested, frozen, freeze-dried and ground in an IKA M20 mill to pass a 0.5 mm sieve and stored at -80 ºC. Extracts were prepared using 1 g of lyophilized powder to 12 mL of 50% (v/v) hydroethanolic solution as described [46].

In vitro digestion

In vitro digestion was performed as previously described (Jardim e*t al*, submitted). Briefly, pH was adjusted to 1.7, 315 units.ml^{-1} of pepsin was added and incubated at 37 °C, 2 h, 100 rpm. Was added 4 mg.ml^{-1} pancreatin and 25 mg.ml^{-1} bile salts mixture. A segment of cellulose dialysis tubing (cut-off 12 kDa), containing 0.1 M NaHCO3 to neutralize titratable acidity, was placed. After 2 h at 37 °C, the solution inside and outside the dialysis tubing were collected. The soluble materials were applied to C18 solid phase extraction columns (GIGA tubes, 1000 mg capacity, Phenomenex Ltd.), as described in [46, 58]. The (poly)phenol-digested metabolites (PDMs) obtained were concentrated and freeze-dried to suitable phenol concentrations determined by the Folin-Ciocalteau method.

Liquid chromatography mass spectrometric (LC-MS) analysis of (poly)phenolic profile

The samples were dissolved in 1.25 mL water/acetonitrile (80:20, v/v) and 0.4 mL was transferred to a 0.45 mm filter vial. Samples were injected via an autosampler onto a Hypersil Gold (50 mm x 2.1 mm; 1.9 µm, Thermo Scientific) reverse-phase UPLC column at 30°C. The HPLC system consisted of an Accella 600 quaternary pump and an Accella PDA detector, coupled to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific Stafford House Boundary Way Hemel Hempstead). The mobile phases were solution A (0.1% (v/v) aqueous formic acid) and solution B (0.1% (v/v) formic acid in acetonitrile/water (50:50, v/v). The flow rate was 450 µL min^{-1}, and the gradient was 0 min; 97% A, 0-3 min; 85% A, 3-7 min; 75% A, 7-10 min; 50% A, 10-13 min; 50% A, 13-14 min 0% A, 14-16 min 0% A, 16-17 min 97% A, 17-20 min 97% A). The PDA detector range was 200-600 nm. Mass

spectrometer mass range was *m/z* 80-2000 with alternative full scan MS and MS/MS data-dependent scans in negative mode [32]. Components were identified using PDA maxima, *m/z* and MS^2 fragmentation data.

Yeast transformation and plasmids

BY4741 (*MAT*a; *his3Δ1*; *leu2Δ0*; *met152Δ0*; *ura32Δ0*) cells were transformed using lithium acetate standard method [59]. The empty plasmids pRS426GAL, p413-GPD or p415 were used as control [60]. The pRS316-GFP-*ATG8* plasmid was a gift from Prof Yoshinori Oshumi (National Institute for Basic Biology, Okazaki, Japan) and was used to sub-clone GFP-*ATG8* as well as the endogenous *ATG8* promoter into the *SacI* - *XhoI* sites of p415 plasmid [34]. Other plasmids used were previously described: p426GAL-aSyn-GFP and p426GAL-aSyn carrying the human gene of aSyn with or without a C-terminal fusion to GFP, under the regulation of *GAL1* inducible promoter [19]; p413-GPD-GFPu expressing an unstable GFP under the regulation of *GPD* constitutive promoter [19].

Yeast growth and compound testing

Cells in *log* growth phase were obtained using synthetic complete (SC) medium [0.67% (w/v) yeast nitrogen base without amino acids (Difco), 1% (w/v) raffinose and 0.79 $g.L^{-1}$ complete supplement mixture (CSM) (QBiogene)], 200 rpm at 30 °C [23]. aSyn expression was induced by growing cells ($OD_{600\ nm}$ 0.2) in SC selective medium 1% (w/v) galactose (aSyn ON) not supplemented or with 62 µg $GAE.mL^{-1}$ leaf or fruit PDMs, for 6 h at 30 °C, 200 rpm. For spot assays $D_{600\ nm}$ of treated cells was set to 0.1±0.005 and 1:10 serially dilutions were prepared [23]. Then, 4 µL of each dilution was spotted in solid SC solid selective medium 1% (w/v) galactose and incubated at 30 °C, 42 h. Images were acquired using Chemidoc™ XRS and Image Lab software. For clearance experiments, after 6 h of aSyn expression induction (with or without PDM treatment), cells were centrifuged, washed in PBS, resuspended in 2% (w/v) glucose SC liquid medium (aSyn expression OFF) and incubated at 30 °C, 200 rpm, 18 h. aSyn levels were determined by western blotting at 6 h of induction (corresponding to 0 h of clearance) and 18 h of clearance.

H4 cell culture and transfections

Human H4 neuroglioma cells (gift from Dr. Bradley T. Hyman, Harvard Medical School) were maintained at 37 °C in OPTI-MEM I (Gibco, Invitrogen, Barcelona, Spain) supplemented with 10% (v/v) fetal bovine serum and seeded at 80,000 cells.cm^{-2} density 24 h prior to transfection. Cells were transfected as previously described with pcDNA3.1-aSynT and pcDNA3.1-Synphilin-1 [41, 42, 61]. For H_2O_2 toxicity assay cells were treated with 2 μg GAE. mL^{-1} *A. unedo* leaf PDM for 16 h, the medium was removed and cells were treated with 600 μM H_2O_2 for 6 h. To evaluate the effect of PDMs in aSyn aggregation, 24 h after transfection cells were treated with 2 μg GAE. mL^{-1} *A. unedo* leaf PDM for 16 h.

Fluorescence microscopy

To determine the percentage of yeast cells with aSyn inclusions, cells were grown as described above and GFP fluorescence was visualized, the percentage of cells presenting aSyn inclusions was determined by counting at least 800 cells for each treatment. Transfected H4 cells were fixed and permeabilized with methanol and blocked in 1.5 % (v/v) normal goat serum in PBS for 1 h. Cells were incubated with primary antibody overnight at 4 °C (mouse anti-aSyn; BD Transduction Laboratories, San Jose, CA, USA) followed by secondary antibody incubation for 1 h (goat anti-mouse IgG-Alexa488, Invitrogen Corporation, Carlsbad, CA, USA). For mitochondria assays, cells were labeled with 200 nM MitoTracker Red FM (Invitrogen), for 30 min at 37 °C protected from light. DAPI was used to stain the nucleus. The proportion of cells with aSyn inclusions within the population was then determined by counting at least 100 cells per condition. Slides were subjected to fluorescence microscopy with a Zeiss Axiovert 200 M Widefield Fluorescence microscope and the counting was performed using ImageJ software.

Western blotting

For aSyn and GFP-Atg8 quantification total yeast protein extraction was performed using the method described [62]. Atg8 induction was quantified by the determination of the fold increase of total GFP signal (GFP-Atg8 and free GFP signal, detected with anti-GFP) normalized to PGK; autophagic flux was quantified by measuring the vacuolar degradation of the Atg8 domain reporter (ratio of free GFP to total GFP signal) [35]. For aSyn quantification

in H4 cells, cells were lysed with NP-40 lysis buffer in the presence of protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany), western blot was performed following standard procedures. Antibodies used: aSyn (BD Transduction Laboratories, San Jose, CA, USA), GAPDH (Ambion, Cambridgeshire, UK), GFP (Antibodies Incorporated, Davis, CA, USA), PGK (Life Technologies, PaisleyUK).

Sucrose gradients

Total protein was obtained from cells expressing aSyn and applied on a 5 to 30% (w/v) sucrose gradient as described before [31, 63]. Fractions were collected, precipitated for 4 h at 4 ºC in trichloroacetic acid, washed in acetone three times and suspended in protein sample buffer (0.5 M Tris-HCl, pH 6.8, 10% (w/v) glycerol, 0.1% (w/v) SDS, 6 mM bromophenol blue). Proteins were resolved by SDS-PAGE, the estimation of the molecular sizes for each fraction was previously described [64].

Flow cytometry (FCM)

FCM was performed in a FACS BD LSR Fortessa, equipped with the *695/40 BP and the 685 LP, as previously described* [23]***.*** ROS was determined in cells transformed with the aSyn encoding plasmid or the empty plasmid, incubated with 50 µM DCFHDA (2',7'-dichlorfluorescein-diacetate) or 30 µM DHE (dihydroethidium), for 15 min at 30 °C, 200 rpm. For cell viability cells transformed with the aSyn-GFP encoding plasmid or the empty plasmid were incubated with PI 5 $\mu g.mL^{-1}$, for 15 min at 30 °C, 200 rpm. To study the proteasome, cells were transformed with the aSyn and GFPu encoding plasmid or the respective empty plasmid. For mitochondria assays in H4 cells, cells were labeled with 200 nM MitoTracker Red FM, for 30 min at 37 °C. Data analysis was performed using FlowJo software. A minimum of 10000 events were collected for each experiment. Results were expressed as median fluorescence intensity (MFI) of a molecule.

Real-time PCR

Quantitative real-time PCR (qRT-PCR) analyzes was performed as described in [38]. The oligonucleotides listed in Table S1 were used to evaluate expression of *SOD1*, *SOD2*, *HAP4*, *LHS1*, *KAR2*, *HRD1*, *GSH1*, *GLR1*, *RPN4*, *SIR2*, and *ATG8* genes. Relative standard curves were constructed for each gene, using triplicate serial dilutions of cDNA. The relative

expression of the genes was calculated by the relative quantification method with efficiency correction, using Applied Biosystems® ViiA™ 7 software. *ACT1*, *PDA1* and *PGK* were used as internal standards and for the normalization of mRNA expression levels [38]. The results were expressed as fold change mRNA levels relative to the control condition and normalized to the reference genes (relative expression).

Glutathione (GSH) and glutathione disulphide (GSSG) quantification

Yeast cells were grown as previously described and GSH and GSSG were quantified by HPLC after sample derivatization, as described in [58].

Statistical analysis

The results reported in this work are the average of at least three independent biological replicates and are represented as the mean ± SD. Differences among treatments were assessed by analysis of variance with Tuckey HSD (Honestly Significant Difference) multiple comparison test ($\alpha = 0.05$) using SigmaStat 3.10.

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Table S1

Gene		Sequence	Eff (%)
GLR1	F	CGACCGGTGGAAAGGCTATT	106
	R	CAGCGCCAACAACAACAACT	
RPN4	F	GCTTCGATACCCCCACAACA	107
	R	TCTATCGTTGGCCGTTGCTT	
HAP4	F	TTCTACTACAGGCCTCCGCT	104
	R	TGGTTGGTATTTGGGGCGAT	
ATG8	F	AATATCTAGTTCCTGCTGACC	108
	R	CCGTCCTTATCCTTGTGTT	
SIR2	F	CGAACTTCCACTATGCCCGT	100
	R	GGAGGCCTTTCCGACATTGA	
KAR2	F	GTCCAAGCCACTTCTGGTGA	100
	R	CATTTGGCTGGACAAGGCAC	
LHS1	F	AGGGTCTTTAGCAGCCGTTT	100
	R	TTTTGCCTCTGGTGTGAGCA	
SOD1	F	CGAGCCAACCACTGTCTCTT	97
	R	ACGTTACCCATGTCACCGAC	
SOD2	F	ACATCAAGTTCCATGGCGGT	104
	R	CTGCTCGTCGATTGCCTTTG	
GSH1	F	ATGGGCTGTTCGTGCTTACA	112
	R	AACGAACATCTTGGTCGGCT	
HRD1	F	AACAGCTCGACGACACTCTTGTCA	85
	R	TCCACGTCTGCTGGTTTGGAGAAT	
HSP26	F	CTACGCACCAAGACGTCAGT	100
	R	ACCAGATGGGAACAGGGACA	
ALG9	F	CGGGAAGCTTGCTCCTGTAT	82
	R	CTAGCACAGGCAGTGGGAAA	
PGK	F	GTGCCAAGGTTGCTGACAAG	72
	R	GAACGATTTCAGCACCAGCC	
PDA1	F	CTGTTGGTCAGGAGGCCATT	65
	R	GCATGGAACCACCCTTACCA	

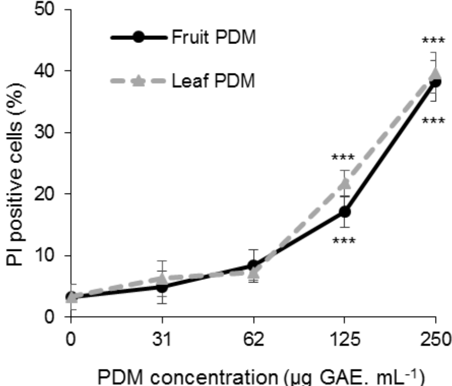


FIGURE S1. *A. unedo* Leaf and Fruit PDM toxicity in control cells. Yeast cells were treated with the indicated concentrations of PDM for 6 h, in galactose liquid medium and cell viability was assessed by flow cytometry using propidium iodide (PI). Values represent the mean ± SD of three independent experiments. Statistically significant differences between the indicated treatments and cells not treated with PDM are shown, ***$p<0.001$.

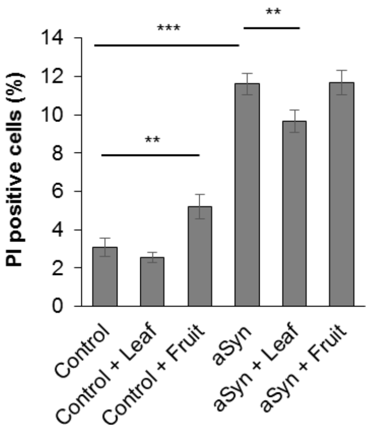


FIGURE S2. *A. unedo* leaf PDM reduce aSyn-induced toxicity. Yeast cells transformed with a plasmid encoding for aSyn or with the empty vector (control), were treated with 62 µg GAE.mL^{-1} of leaf or fruit PDM for 6 h, in galactose liquid medium. Cell viability was assessed by flow cytometry using propidium iodide (PI). Values represent the mean ± SD of three independent experiments. Statistically significant differences between the indicated treatments are shown, **$p<0.01$, ***$p<0.001$.

Discussion

Neurodegenerative diseases are age-related disorders that present heterogeneous symptoms, affect diverse brain regions, and are associated with a variety of molecular alterations that suggest a highly complex etiology. This poses tremendous challenges for the identification of relevant drug targets and the development of effective therapies. Amongst neurodegenerative diseases PD is the second most common, after AD, with a prevalence of 1-2% after the age of 65 years [1]. Current therapies for PD are only symptomatic, restoring the dopamine levels in the brain without lessening the primary causes of this illness [2].

The deeper we investigate the mechanism of disease, the closer we become to identifying useful drug targets and to designing drugs with pleiotropic effects, capable of acting not only in one of the pathological events but in the multitude of events that lead to disease, ultimately restoring the homeostasis of the brain and fighting neurodegeneration.

The cellular events leading to PD comprise the misfolding and aggregation of aSyn [3-5], oxidative stress [6-8], mitochondrial dysfunction [8-12], deficient dopamine release [2], impairment of the cellular quality control systems [4, 6, 13-18], deficient vesicular and axonal transport [11, 15, 19, 20, 21], ER-to-Golgi trafficking impairment [10, 22, 23] and, ultimately, neuronal death [15, 24, 25].

Cytoplasmic inclusions primarily made of fibrilar aSyn, known as LBs are a major hallmark of PD [26]. Evidence suggests that these inclusions may actually constitute a protective mechanism which neurons developed to prevent the accumulation of toxic aSyn intermediary oligomeric species [27, 28,52, 53,] . One explanation is that once oligomers accumulate in aggregates they are no able to interact with membranes and organelles, diminishing the neurotoxic properties of oligomers. The kinetics of oligomer formation is likely modulated by various cellular components, ageing and external stimuli [29]. Nevertheless, the way by which misfolding results in disease and the players involved in the aggregation of aSyn are not completely understood.

Despite intense research, and the vast knowledge accumulated over the years, a clear example that PD pathology still poses challenges comes from the lack of understanding concerning the role of phosphorylation in aSyn aggregation.

Remarkably, in LBs of patients with synucleinopathies, 90% of aSyn is phosphorylated in the S129 residue [30]. Nonetheless, the consequences of this post-translational modification in PD are still unclear. While some studies favour the hypothesis that pS129 is protective others argue that it potentiates toxicity. In *Drosophila* pS129 accelerated neuronal loss [31]. Contrarily, in rat primary neurons and *C. elegans* the overexpression of PLK2 was protective [32]. Additionally, the S129A phospho-resistant aSyn lead to increased toxicity in the brain of rats [33, 34]. We observed that blocking aSyn phosphorylation on S129 resulted in an exacerbation of aSyn toxicity, with concomitant formation of higher molecular weight species of aSyn[35]. Furthermore, the unphosphorylatable aSyn failed to induce autophagy, a major pathway responsible for the degradation of larger aSyn inclusions and fibrils [14, 36], resulting in a deficient degradation of aSyn [35]. The impairment in autophagy could be a consequence of the known adverse effect of aSyn oligomers on vesicle formation [37, 38], and is corroborated by the ER-to-Golgi vesicular trafficking defects (Fig. 1). Furthermore, ER is a known source of membranes for autophagy [39], if unphosphorylated aSyn disrupts ER function leading to ER-stress it could result in the debilitated formation of autophagosomes, and the concomitant impairment of autophagy induction. Another hypothesis is that S129 phosphorylation might constitute a switch to sense and induce the protein quality control by autophagy. Accordingly, in HD, phosphorylation of mutant huntingtin was found to precede and regulate additional post-translational modifications, defining the fate of the protein [40]. In particular, phosphorylation mediates the subsequent acetylation of the protein, which in turn targets huntingtin to degradation by autophagy [41].

The relationship between aSyn phosphorylation and its clearance has been recently investigated, and over-expression of polo-like kinase 1 (PLK2) resulted in selective clearance of pS129 by autophagy [42]. Thus, the increased aSyn phosphorylation observed in PD brains may constitute a protective mechanism. It is tempting to speculate that pS129 functions as a trigger for subsequent post-translational modifications (as ubiquitination) and for protein degradation. As such, the accumulation of phosphorylated aSyn in PD could be explained by the impairment of the clearance pathways, with concomitant accumulation of phosphorylated along with ubiquitylated aSyn. To explore this hypothesis

further, the interplay between the different post-translational modifications and the protein clearance pathways needs to be scrutinized. Nevertheless, the work presented here constitutes a milestone in the understanding of aSyn phosphorylation, toxicity, aggregation and clearance, shedding light into PD pathology.

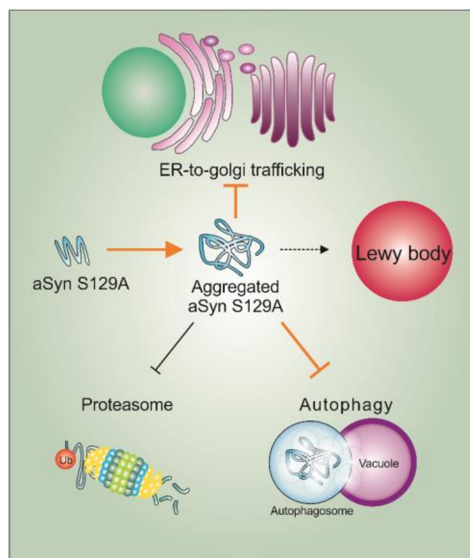


Figure 1: Effect of S129 phosphorylation on aSyn pathobiology. The *SNCA* gene was integrated in the genome of yeast to allow the stable expression of alpha-synuclein (aSyn). Upon aSyn WT expression the residue S129 was phosphorylated by endogenous kinases in yeast. Using an aSyn mutant, where the serine was substitute by an alanine, we studied the effect of S129 phosphorylation on aSyn pathobiology**.** The unphosphorylated S129A caused cellular toxicity, promoted its aggregation leading to higher molecular weight aggregates, and exacerbated the endoplasmatic reticulum (ER)-to-Golgi trafficking defects. Importantly, blocking phosphorylation resulted in impaired degradation of aSyn due to a defect in the induction of the autophagic pathway (orange arrows). This suggests a beneficial role for aSyn S129 phosphorylation in PD and highlights the importance of this PTM in the regulation of protein clearance by autophagy.

Nevertheless, it is also crucial to search for modulators of the cellular pathways involved in neurodegeneration, in order to developed efficient therapeutic approaches. Oxidative stress plays a central role in PD [6-8, 43, 44], however it is not completely clear if it is a critical initiator of the disease or a consequence and a subsequent enhancer of PD. The ubiquity of oxidative stress in human diseases has increased the interest in the

identification of molecules able to reduce or revert oxidative stress. (Poly)phenols, which were for many years regarded as strong radical-scavengers and antioxidants, became attractive subjects of study, and their diverse bioactivities began to emerge. Nowadays, between the health promoting properties of these compounds are their capacity to directly interact with receptors or enzymes involved in signal transduction, such as protein kinase and lipid kinase signalling pathways [45]; metal chelating properties [46]; modulation of signalling pathways related with neuronal survival and differentiation [45, 47]; inhibition of neuropathological processes [48, 49]; and regulation of mitochondrial function [50, 51].

We explored Portuguese plant species to study the pharmacological and nutritional potential of (poly)phenols in PD [52]. Remarkably, (poly)phenols obtained from the leaves of *C. album* presented the most promising properties for a pharmacological approach. *C. album* is a plant that occurs mainly on the Atlantic coast of Portugal and was poorly characterized [54, 53]. (Poly)phenols from *C. album* protected yeast cells from aSyn induced toxicity, reduced oxidative stress, diminished aSyn inclusions, and promoted autophagy flux, which was impaired by aSyn [52]. Furthermore, *in vitro* studies revealed that its (poly)phenols inhibited aSyn fibrillization and stabilized aSyn oligomeric species. Surprisingly, these oligomers were not toxic in a human cell model of PD.

Experimental findings favour the formation of neurotoxic oligomers as a key pathological event in synucleinopathies [54, 55]. Oligomeric species have a negative impact on the UPS machinery and on mitochondrial function [56], and they also trigger ER stress-responses [23, 57] and caspase activation [58, 59]. The equilibrium between soluble and aggregated species can be shifted towards the former or the latter by using small molecule inhibitors or enhancers of aggregation, respectively. The stabilization of the native structure of proteins or the promotion of aSyn fibrillization have been proposed as appealing intervention strategies. These approaches would prevent the formation of the toxic oligomers and intermediates.

Notwithstanding, our study proposes a different strategy. It evidenced that (poly)phenols promoted the formation of non-toxic oligomeric species. Hence, not all oligomers appear to be toxic, and promoting the formation of this type of oligomers can actually be beneficial for cells. Interestingly, these SDS-resistant dimers seem to be the

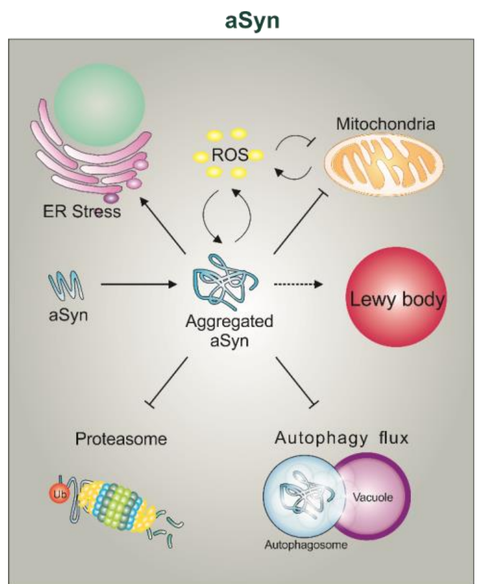
most commonly detected form of aSyn oligomers in denaturing gels in the presence of specific metals and small molecules, such as (poly)phenols [60]. These dimers are stable in the presence of dissociative conditions such as reducing agents, chemical denaturants and boiling, suggesting that they are covalently cross-linked [60, 61], as observed in our study. We postulate that these oligomers are non-propagating dimers [29], since the aSyn fibrillization process is inhibited by (poly)phenols. Moreover, our detergent-solubility assay, revealed that (poly)phenols increased the insolubility of aSyn. These observations are consistent with those observed in *Drosophila*, where reduced aSyn toxicity was correlated with an increase in detergent-insoluble aSyn [62], and suggests this may constitute a defence mechanism.

(Poly)phenols are also considered important molecules in the context of human nutrition and are explored in the prevention of human diseases, since they are widely distributed in fruits and vegetables [63-65]. To investigate (poly)phenols in the context of nutrition, they were submitted to an *in vitro* digestion procedure, which mimics the upper gastro-intestinal digestion. This procedure has been extensively used to mimic polyphenols digestion as revised by European COST action INFOGEST [66] and its main outputs were validated against the human *in vivo* digestion [67]. From the nutritional perspective, the use of digested metabolites is a substantial improvement. However, the model used does not mimic the complete bioavailability. It misses the active transport in the gut, their metabolism by the microbiota of the colon, and the conjugation of metabolites. Nevertheless, it is currently extremely difficult to conceive a model accomplishing all these features.

In a previous study we reported the effect of digestion in the chemical profile of fruits and leaves from *C. album*, *A. unedo*, *G. biloba* and *R. idaeus* (Jardim *et al,* unpublished). Notably, the *in vitro* antioxidant capacity of these species was lost upon simulated digestion, reinforcing how limited *in vitro* assays are to predict the antioxidant capacity of foods in nutritional studies. Digested metabolites from *A. unedo* leaves protected yeast cells from an oxidative insult (Jardim *et al,* unpublished), protected from aSyn toxicity, reduced the formation of aSyn inclusions and the size of the aggregates. The observed cytoprotection was related with the regulation of pathways intertwine with

protein misfolding, such as oxidative stress, mitochondria dysfunction, ER-stress and quality control systems (proteasome and autophagy) (Fig. 2).

Notably, although (poly)phenols suffer alterations during gastro intestinal digestion and their antioxidant capacity is lost, they present beneficial effects in models of neurodegenerative disorders, suggesting other molecular mechanism conferring protection.



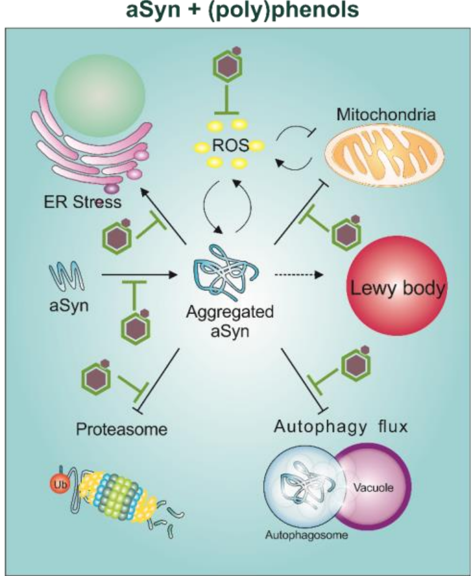


Figure 2: Effect of (poly)phenol-digested metabolites (PDM) from *A. unedo* leaves on aSyn pathobiology. aSyn WT was expressed in yeast, recapitulating several PD hallmarks such as cellular toxicity, protein aggregation, ER stress, oxidative stress promoted by reactive oxygen species (ROS), mitochondria and protein clearance impairment. The treatment with PDMs increased cell viability, which can be related to the observed inhibition of protein aggregation, reduction of ROS and increased mitochondrial activity. We also observed that PDM promoted the proteasome and autophagic function, ultimately reducing ER stress.

A feature of PD that connects the three studies presented here is the impairment of cellular quality control pathways. Blocking S129 aSyn phosphorylation impaired the induction of autophagy, increasing aSyn toxicity. On the other hand, *C. album* (poly)phenols and *A. unedo* digested metabolites induced the autophagic flux, impaired by the expression of aSyn. This works opens avenues to study the connection between

(poly)phenols, aSyn phosphorylation and autophagy, clearly a subject that must be explored. Studies reported that (poly)phenols can act on several kinases known to be involved in aSyn phosphorylation [68]: catechins acted on PLK1 [69]; resveratrol, EGCG and coumarins on casein kinase 2 (CK2) [70, 71]; EGCG on dual-specificity tyrosine-(y)-phosphorylation regulated kinase 1A (Dyrk1A) [72], an finally quercetin and resveratrol on spleen tyrosine kinase (Syk) and sarcoma (Src)-family kinases [73]. Importantly, PLK1 [74] and CK2 [75] are described to phosphorylate aSyn at residue S129, Dyrk1A at S87 [76], Syk at Y125, Y133 and Y136 [77], and Src at Y125 [78]. However, the studies with (poly)phenols did not explored the phosphorylation state of aSyn.

(Poly)phenols were also described to modulate phosphorylation signalling cascades involved in cellular survival, quercetin inhibited phosphoinositide 3-kinase (PI 3-kinase) [79], quercetin metabolites modulated Akt/protein kinase B (Akt/PKB) [80], flavonoids inhibited protein kinase C (PKC) [81], and quercetin acted on mitogen activated protein (MAP) kinase [82, 83]. The modulation of these pathways alter the phosphorylation state of the target molecules and modulate gene expression interfering with cellular functions, with implication for neurodegeneration. For instance resveratrol protection was shown to be mediated by activation of autophagy through AMPK-SIRT1 pathway on PD cellular models [84].

Importantly, the yeast prove to be a versatile model to study protein quality control pathways and phosphorylation in PD. We exploited various yeast models based on the heterologous expression of aSyn according to the goals of our studies. Depending on the expression system, these models presented different phenotypes, given that this affects the level of aSyn expression [15, 23, 85]. In the first study, the *SNCA* gene was integrated in the genome of yeast to allow the stable expression of aSyn, resulting in a slight toxic WT aSyn expression. In the second and third studies, a multi-copy vector was used to express WT aSyn in yeast, this resulted in a moderate aSyn toxicity, allowing the identification of toxicity rescuers. The diversity of yeast models, and conditions used, explain the divergence between autophagy results. It is also important to consider the growth phase of the cell when studying autophagy, as other studies reported that induction of autophagy by aSyn is toxic in aged cells [13].

A reasonable explanation to aSyn toxicity mediated by autophagy induction, is that the excessive activation of a dysfunctional autophagy will lead to a loss of selectivity, resulting in the trapping of functional competent proteins and organelles in autophagosomes. Ultimately, leading to a loss of function and cell toxicity. Thus, the beneficial or detrimental role of autophagy should be studied having in consideration its functionality and selectivity, as well as the size and nature of the aSyn aggregates. Furthermore, (poly)phenols and S129A studies provided valuable hints on how autophagy can be modulated to overcome the protein overload in the cell.

Overall, the goal for PD is to identify useful drug targets and to development more efficient therapies. To achieve these ambitious goals we need to gain extensive knowledge of the molecular mechanism of the disease and search for health promoting molecules. Looking ahead, the work presented in this thesis provides the framework for future studies. It is tempting to study aSyn post-translational modifications, as well as the effect of (poly)phenols, in animal models.

Regarding aSyn phosphorylation it will also be important to understand the interplay between the several post-translational modification and the protein clearance pathways, in order to understand i) how (un)phosphorylated aSyn is recognized by autophagy and ii) how (un)phosphorylated aSyn affect autophagy in particular autophagosomes formation. In what concerns (poly)phenols it would be interesting to address iii) how are degraded the aSyn species formed in the presence of (poly)phenols, and iv) how are these oligomers different from the toxic oligomers. To answer this questions NMR, crystallography or electron microscopy could be useful to understand the changes in the structure of aSyn promoted by (poly)phenols. Whether (poly)phenols effect on autophagy is mediated by modulation of phosphorylation and interfere or not with aSyn phosphorylation levels remains unclear, and is a subject currently being investigated by us using a phosphoproteomic approach.

For a rational application of (poly)phenols for human nutrition or as pharmaceuticals it is also important to assess its pharmacokinetic and determine its absorption, metabolism, tissue and organ distribution, as well as excretion *in vivo* and perform nutritional human intervention studies. In a more applied perspective, it would be

remarkable to do a bio-guided fractionation of *C. album* to understand the main compounds responsible for the protection observed and to study the possibility of doing a nutritional formulation with *A. unedo* leaves.

The work presented in this thesis explored PD pathobiology to gain insight into the molecular mechanism of disease, shedding light into new potential drug targets and strategies based on (poly)phenols. However, in science every answer raises new questions, and as Carl Sagan once said “somewhere, something incredible is waiting to be known”. The cell is microscopic but contains a universe full of galaxies in relentless transformation, waiting to be discovered.

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