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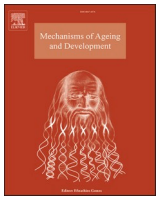

A geroscience approach for Parkinson's disease: Conceptual framework and design of PROPAG-AGEING project

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ABSTRACT

Advanced age is the major risk factor for idiopathic Parkinson's disease (PD), but to date the biological relationship between PD and ageing remains elusive. Here we describe the rationale and the design of the H2020 funded project "PROPAG-AGEING", whose aim is to characterize the contribution of the ageing process to PD development. We summarize current evidences that support the existence of a continuum between ageing and PD and justify the use of a Geroscience approach to study PD. We focus in particular on the role of inflammaging, the chronic, low-grade inflammation characteristic of elderly physiology, which can propagate and transmit both locally and systemically. We then describe PROPAG-AGEING design, which is based on the multi-omic characterization of peripheral samples from clinically characterized drug-naïve and advanced PD, PD discordant twins, healthy controls and "super-controls", i.e. centenarians, who never showed clinical signs of motor disability, and their offspring. Omic results are then validated in a large number of samples, including in vitro models of dopaminergic neurons and healthy siblings of PD patients, who are at higher risk of developing PD, with the final aim of identifying the molecular perturbations that can deviate the trajectories of healthy ageing towards PD development.

1. Introduction

In 2016, 6.1 million people suffered from Parkinson's Disease (PD) worldwide (Dorsey et al., 2018). Among neurodegenerative diseases, PD is the second most common (after Alzheimer's Disease) and the one that displayed the largest increase in prevalence, which more than doubled from 1990 to 2016 (Dorsey et al., 2018). This increase in the number of PD patients is largely, although not exclusively, sustained by the ageing of the population, as advanced age is acknowledged to be the major risk factor for developing PD (Reeve et al., 2014). Accordingly, PD is uncommon before 50 years and its prevalence steeply increases after 65 years, peaking between 85 and 89 years of age (Bennett et al., 1996; Dorsey et al., 2018; Pringsheim et al., 2014).

In spite of this epidemiological evidence, to date, the biological relationship between PD and ageing remains elusive. This is at least in part due the paucity of experimental settings specifically aimed at investigating PD in the framework of the ageing process, in particular when studies performed on humans are considered (Pang et al., 2019).

With the aim of filling this gap, the European Consortium PROPAG-AGEING ("The continuum between healthy ageing and idiopathic Parkinson Disease within a propagation perspective of inflammation and damage: the search for new diagnostic, prognostic and therapeutic targets"; grant agreement 634821) has been established in the framework of the European call PHC-01-2014 (Understanding health, ageing and disease: determinants, risk factors and pathways). PROPAG-AGEING Consortium is highly interdisciplinary and gathers together 9 participants from high ranking academic and non-academic institutions throughout Europe, with a well-established expertise at both clinical and molecular level (Table 1).

PROPAG-AGEING implements a Geroscience approach for the study of PD. Geroscience is an interdisciplinary field that emphasizes the common mechanisms (operating at the level of molecules, cells, organs, systems and ecosystems) shared by physiological ageing and age-related diseases (ARDs) (Kennedy et al., 2014). According to Geroscience ARDs, including PD, are interpreted as the result of a local or systemic accelerated ageing process. This approach can contribute to the study of PD at different levels: i) at a mechanistic level, by investigating whether the molecular/cellular perturbations characteristic of the ageing process occur, possibly in a more pronounced or accelerated fashion, also in PD; ii) at a diagnostic/prognostic level, by investigating whether the biomarkers used to track ageing are also able to detect the onset or the progression of PD; iii) at the therapeutic level, by investigating whether anti-ageing drugs and interventions are potentially effective also in the treatment and prevention of PD.

In this framework, the main goal of PROPAG-AGEING is to identify the molecular/cellular perturbations deviating the phenotype of elderly subjects from a physiological decline to clinically overt PD. The design of the project is thus specifically implemented in order to track the trajectories of healthy ageing and of PD, using the same analytical approaches.

In the next paragraphs we will summarize the rationale at the basis of PROPAG-AGEING, which justifies the use of a Geroscience approach to study PD, and we will describe the design of the project and its implementation by the members of the Consortium.

Table 1
PROPAG-AGEING Consortium.

Partner	Acronym	Country
AZIENDA UNITA' SANITARIA LOCALE DI BOLOGNA	AUSL-ISNB	Italy
UNIVERSITY COLLEGE LONDON	UCL	United Kingdom
UNIVERSITAETSMEDIZIN GOETTINGEN - GEORG-AUGUST-UNIVERSITAET GOETTINGEN - STIFTUNG OEFFENTLICHEN RECHTS	UMG-GOE	Germany
SERVICIO ANDALUZ DE SALUD	SAS	Spain
PERSONAL GENOMICS SRL	PG	Italy
THE CHANCELLOR, MASTERS AND SCHOLARS OF THE UNIVERSITY OF UCAM CAMBRIDGE	UCAM	United Kingdom
CONSORZIO INTERUNIVERSITARIO RISONANZE MAGNETICHE DI METALLOPROTEINE	CIRMMP	Italy
KAROLINSKA INSTITUTET	KI	Sweden
ALMA MATER STUDIORUM - UNIVERSITA DI BOLOGNA	UNIBO	Italy

2. PROPAG-AGEING rationale: the continuum between ageing and PD and the propagation hypothesis

Ageing is the result of a complex interplay between ontogenetic programs, genetic influences, life course environmental exposures and stochastic events (Cevenini et al., 2010; Dorsey et al., 2018; Franceschi et al., 2020) that concur to the high heterogeneity of phenotypes observed among the elderly (Franceschi et al., 2017b). Indeed, if on the one hand some persons develop age-related disease like PD, on the other some persons can reach the extreme limit of life in good health, i.e. the centenarians. Amongst these two extremes, there is a continuum of intermediate phenotypes including persons with subclinical manifestations of diseases more or less pronounced. This implies that, for a determined age range, it is difficult to classify an individual as absolutely healthy and that each individual follows his specific ageing trajectory (Fig. 1).

Different authors have identified a limited but highly interconnected set of hallmarks of ageing that is also shared with ARDs and therefore contributes to their risk (Kennedy et al., 2014; López-Otín et al., 2013). More specifically, features shared between ageing and PD include neuroanatomical changes, accumulation of α-synuclein, cell senescence and changes in glial environment, mitochondrial dysfunction, oxidative and nitrative stress, impairment in proteasome and lysosome functions, gut microbiome deregulation and alteration of glial environment,

among others (Calabrese et al., 2018).

Histopathologically, PD is characterized by a patterned, preferential loss of dopaminergic neurons (DA) in the *pars compacta* region in the *substantia nigra* (SN) and the presence of intracellular inclusions, called Lewy bodies, containing α-synuclein aggregates (Braak et al., 2003). A decrease in the number of DA neurons, together with other pathological changes in this brain region, is also observed during physiological ageing, without clinical symptoms suggesting PD. The course of neurodegeneration is gradual and slow and from the initiation of the neuronal damage and death to the PD diagnosis there is a long-time lag. Buchman et al. considered a large cohort of 744 healthy elderly (average age 88.5 years) without a clinical diagnosis of PD at death and found that about 1/3 showed a mild to severe neuronal loss within the *substantia nigra*, 17 % showed Lewy bodies and 10 % showed both these pathological features (Buchman et al., 2012). This study plasticly exemplifies the linear relationship between the concomitant increase of life expectancy and PD case prevalence. A PD-like pathology is likely much more common in the apparently healthy elderly population, but the vast majority of cases do not survive enough to pass the quantitative cutoff of neurodegeneration and experience the onset of clinically overt disease (Burke and O'Malley, 2013; Cheng et al., 2010). Other studies reported an age-dependent increase in α-synuclein in the brains of healthy aged humans (Chu and Kordower, 2007; Xuan et al., 2011) as well as in the brain and in the enteric nervous system of animal models (Chu and Kordower, 2007; Li et al., 2018; Phillips et al., 2009), supporting the Braak hypothesis of gut-to-brain spreading of α-synuclein (Braak et al., 2003; Kim et al., 2019). Experiments in Rhesus monkeys also reported a selective age-dependent decline in DA neurons in the ventral tier of SN, similarly to what happens in PD (Kanaan et al., 2007).

α-synuclein clearance from the cytosol is performed by the ubiquitin-proteasome and lysosome-autophagy system – advancing age and the PD disease being processes both associated with decreased activity in these systems (Collier et al., 2011). Accordingly, histological analyses in post-mortem PD tissues identified impaired proteasomes and lysosomes (McNaught et al., 2003).

Increased oxidative and nitrative damage is a hallmark of PD in brain tissue (Dias et al., 2013). Reactive oxygen and nitrogen species (ROS and RNS) are produced by mitochondria as side-products of aerobic respiration, and their lifelong accumulation substantially contributes to ageing (Harman, 1956). Interestingly, accumulation of α-synuclein in mitochondria was found in PD, associated to an impairment in the activity of electron transport chain complex I and to an increase in oxidative stress and in ROS production (Devi et al., 2008). The boost in the ROS generation is closely related to the higher inflammation level reported both in ageing and PD (Guo et al., 2018; Vida et al., 2014). The contribution of peripheral inflammation and neuroinflammation to PD pathogenesis has been extensively summarized elsewhere (Caggiu et al., 2019; Calabrese et al., 2018; Collins et al., 2012; Qin et al., 2016). Here we will underline some general aspects of this phenomenon, relevant in the context of the PROPAG-AGEING project:

2.1. A close relationship exists between PD-related inflammation and inflammaging

Inflammaging, i.e. the chronic, low-grade inflammation that occurs during ageing (Calabrese et al., 2018), is regarded as one of the main contributors of ARDs, including neurodegenerative diseases (Furman et al., 2019). As recently conceptualized (Franceschi et al., 2018a) inflammaging is triggered by the accumulation of non-self (pathogens), quasi-self (nutrients and microbiota products) and self (damaged and/or misplaced) molecules that converge on the activation of a limited number of sensors. These sensors promote the activation of a pro-inflammatory response, which concomitantly stimulates an adaptive activation of anti-inflammatory processes (anti-inflammaging).

In the brain, neuro-inflammaging is sustained by a complex interplay between different cellular types, including neurons, microglia, astrocytes and leukocytes that can penetrate the damaged blood-brain barrier (Costantini et al., 2018).

2.2. Inflammaging can propagate locally (cell-to-cell) and systemically (through the blood and lymphatic stream) (Franceschi et al., 2017a)

For example, damaged/misplaced self-molecules produced within the cell can be released by cell necrosis or actively secreted by extracellular vesicles, like exosomes. These pro-inflammatory compounds can affect the microenvironment of the adjoining cells and/or can enter the circulation, stimulating the inflammatory response in distal tissues and organs. The same propagation process applies also to the complex mixture of cytokines and pro-inflammatory molecules released by senescent cells and termed Senescence Associated Secretory Phenotype (SASP) (Acosta et al., 2013; Tchkonia et al., 2013).

Senescent cells have been detected in brains from elderly subjects and patients with neurodegenerative diseases (Baker and Petersen, 2018; Martínez-Cué and Rueda, 2020). In particular, markers of cell senescence have been reported in astrocytes from PD patients, and they have been reported to accumulate as a consequence of the exposure to environmental compounds like Paraquat (Chinta et al., 2018, 2013). The exact mechanisms by which inflammaging is propagated from the periphery to the brain and vice versa are still elusive, but experiments involving heterochronic parabiosis and plasma administration strongly

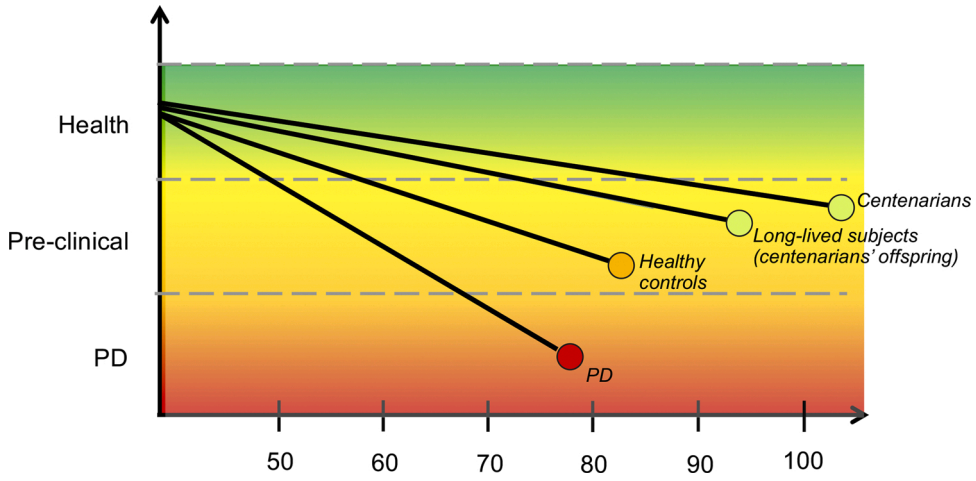

Fig. 1. The continuum between ageing and PD. The continuum is represented as a shade of color from green to red. Each line corresponds to the ageing trajectories of PD, general population (healthy controls) and long-lived subjects (centenarians and their offspring). The colour of circles corresponds to health status at death.

support the propagation hypothesis of inflammaging and indicate that brain ageing is intimately linked to the presence of pro- and anti-ageing molecules in the circulation (Horowitz and Villeda, 2017). A propagatory model of inflammaging has been presented (Franceschi et al., 2018b; Gordleeva et al., 2020), within a conceptualization of the body as a Super-Network (Whitwell et al., 2020).

2.3. Inflammaging is a life-course process

The balance between inflammaging and anti-inflammaging is continuously remodelled during the life of the individual and is the result of the complex interaction between their genetic background and the environment to which they are exposed (starting from the very beginning of life and considering also preconception and in utero exposures) (Franceschi et al., 2007). Both genetic and environmental factors substantially contribute to PD, possibly impinging on the balance between inflammaging and anti-inflammaging. Importantly, an interplay between ageing, genetic predisposition to PD and exposure to chemicals has been reported, supporting the hypothesis that the ageing milieu sustains and amplifies the effects of genetic and environmental factors (Liu et al., 2017; Marder et al., 2015; Pang et al., 2019).

In summary, PROPAG-AGEING rationale is based on two main pillars:

1) The environment feeding PD onset and progression is the elderly physiology, and there is therefore a continuum between healthy ageing and PD. The project assumes PD as totally embedded within the basic molecular and cellular mechanisms of the ageing process, including inflammaging and neuro-inflammaging, among others.
2) Inflammaging, ageing and PD can propagate and transmit both locally and systemically. As a consequence, peripheral biospecimens (like blood, urine and stool) can be investigated not only to identify molecular, cellular and clinical markers of PD, but also to characterize the alterations that trigger the onset and the progression of the disease.

In the next paragraphs, we will discuss the design of PROPAG-AGEING project, which is described in Fig. 2 and Table 2.

3. PROPAG-AGEING design: the cohorts included in the study

As mentioned above, few studies have investigated in the same experimental settings the signatures of ageing and those of PD. The design of PROPAG-AGEING has been specifically implemented in order to fill this gap. The project is based on a large number of human samples deriving from existing multi-center cohorts (that is, collected by the partners before PROPAG-AGEING, in the framework of other national and international projects) and including (Fig. 2):

- *de novo* PD patients, for which clinical characterization and collection of biological specimens have been performed at disease onset, before the dopaminergic therapy, according to the UK Brain Bank Criteria (Gibb and Lees, 1988). The analysis of *de novo* patients is highly informative, as it avoids possible confounding effects associated with the dopaminergic treatment, which is likely to alter the signatures of ageing and PD and to impair the detection of early markers of the disease;
- advanced PD patients;
- monozygotic (MZ) and dizygotic (DZ) twins from the Swedish Twin Registry, overall followed longitudinally for more than 45 years and assessed for lifestyle and place of living, type of work and exposure to potential environmental toxicants. Twin couples discordant for PD have been accurately recorded, and biological samples (blood and sera) have been collected before PD onset (incident cases) and/or after PD onset (prevalent cases);
- healthy control subjects, including sex-, country- and age-matched with PD patients, but also subjects younger and older than PD patients, that allow to track the trajectories of healthy/physiological ageing;
- healthy aged "super-controls", including both thoroughly characterized centenarians who never showed clinical signs of motor disability despite their exceptional lifespan, and their offspring.

In PROPAG-AGEING therefore we will consider a continuum of phenotypes and we will adopt the highly informative strategy of comparing extreme phenotypes (PD patients on one side; centenarians and their offspring, on the other side) (Garagnani et al., 2013; Giuliani et al., 2017) to maximize the possibility to identify PD-specific signatures.

Centenarians can be considered a paradigm of healthy ageing, as they largely avoided or postponed most of ARDs. Interestingly, while dementia is present in a minority of centenarians, PD is not (Arosio et al., 2017; Marcon et al., 2020), suggesting that PD is not an unavoidable result of the ageing process. We and others previously demonstrated that centenarians are characterized by specific clinical, cellular and molecular signatures associated to a healthy phenotype (Collino et al., 2013;

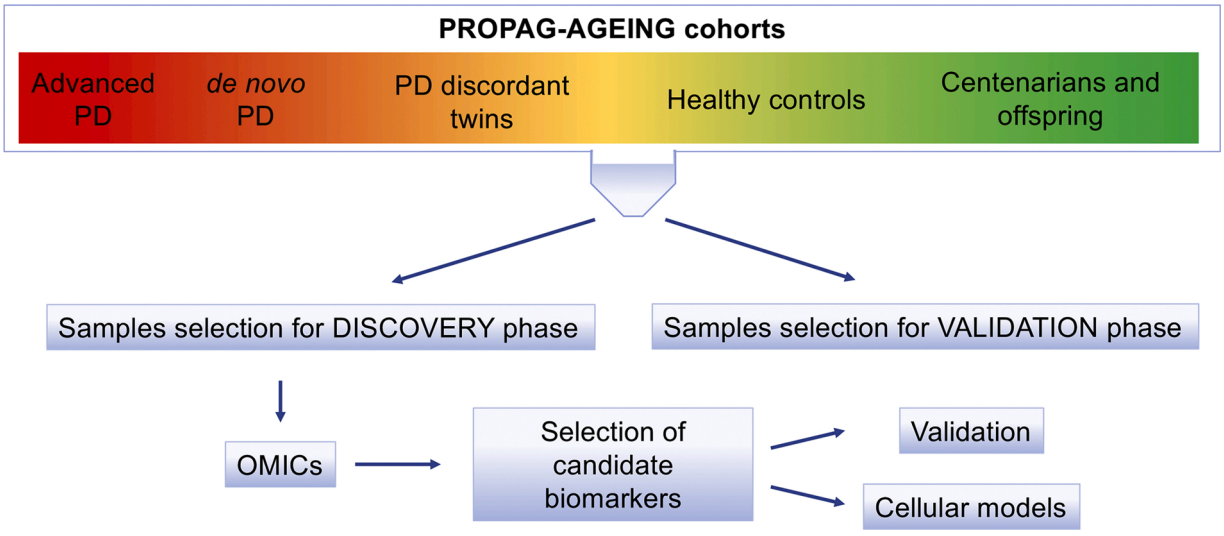

Fig. 2. PROPAG-AGEING design. The cohorts included in the project and the envisaged workflow are reported. The continuum of phenotypes is represented as a shade of colour from red (PD) to green (long-lived subjects).

Table 2
Overview of PROPAG-AGEING analyses. The table reports the analyses foreseen in the project (considering the discovery and the validation phases), the techniques applied, the cohorts available for each analysis and the biospecimen. Finally, it indicates the comparisons and the scientific questions that will be addressed.

Analysis	Techniques	Cohorts	Biospecimens	Comparisons	Scientific questions/Expected results
Genetics discovery (UCL)	Whole genome sequencing	de novo PD, controls (UMG-GOE) advanced PD (AUSL-ISNB) centenarians, controls (UNIBO)	Whole blood	PD (de novo and advanced) vs controls, taking into account the recruitment center	Genetic variants associated to PD
				PD vs centenarians, taking into account the recruitment center	Genetic variants associated to PD and not associated to successful ageing (comparison between extreme phenotypes)
Genetics validation (UNIBO)	iPLEX MassARRAY	de novo PD, controls (UMG-GOE) advanced PD (AUSL-ISNB) advanced PD, controls (SAS) centenarians, controls (UNIBO) PD siblings (UMG-GOE, AUSL-ISNB, SAS)	Whole blood	PD (de novo and advanced) vs controls, taking into account the recruitment center	Genetic variants associated to PD
				PD vs centenarians, taking into account the recruitment center	Genetic variants associated to PD and not associated to successful ageing (comparison between extreme phenotypes)
				Association with risk factors in PD siblings	Genetic variants associated with risk of prodromal PD
Epigenetics discovery (AUSL-ISNB)	Infinium MethylationEPIC (Illumina)	de novo PD, controls (UMG-GOE) advanced PD (AUSL-ISNB) centenarians, centenarians'offspring, controls of different age (UNIBO)	Whole blood	de novo PD vs controls	DNAm changes in early phases of PD not under treatment
				Advanced PD vs controls	DNAm changes in PD under treatment
				PD (de novo and advanced) vs centenarians'offspring	DNAm changes associated to PD and not associated to successful ageing
				Association with age (controls of different age) and with successful ageing (centenarians)	Comparison of DNAm trajectories in healthy/successful ageing respect to PD; epigenetic clocks (accelerated ageing in PD?)
		MZ and DZ twins discordant for PD (KI)		Intra-couple analysis in discordant twins	DNAm changes associated to PD, taking into account the genetic background/environmental exposures
Epigenetics validation (UNIBO)	EpiTYPER MassARRAY	de novo PD, controls (UMG-GOE) advanced PD (AUSL-ISNB) advanced PD, controls (SAS) centenarians, centenarians'offspring, controls of different age (UNIBO) MZ and DZ twins discordant for PD (KI) PD siblings (UMG-GOE, AUSL-ISNB, SAS)	Whole blood	As in the discovery phase of epigenetic analysis	As in the discovery phase of epigenetic analysis
				Association with risk factors in PD siblings	DNAm changes associated with risk of prodromal PD
Transcriptomics discovery (SAS)	RNA-seq	de novo PD, controls (UMG-GOE) centenarians, controls (UNIBO)	Whole blood	de novo PD vs controls	Differentially expressed genes in early phases of PD not under treatment
				de novo PD vs centenarians controls vs centenarians	Differentially expressed genes associated to PD, ageing and successful ageing
Transcriptomics validation (SAS)	Open Array Real-Time PCR system	de novo PD, controls (UMG-GOE) advanced PD, controls (SAS) PD siblings (UMG-GOE, AUSL-ISNB, SAS)	Whole blood	de novo PD vs controls advanced PD vs controls	Differentially expressed genes in early phases of PD not under treatment and in PD under treatment
				Association with risk factors in PD siblings	Differentially expressed genes associated with risk of prodromal PD
miRNomics discovery (PG)	miRNA-Seq	de novo PD, controls (UMG-GOE) centenarians, controls (UNIBO)	Serum	de novo PD vs controls	Differentially expressed circulating miRNA in early phases of PD not under treatment
				de novo PD vs centenarians controls vs centenarians	Differentially expressed circulating miRNA associated to PD, ageing and successful ageing
		MZ and DZ twins discordant for PD (KI)		Intra-couple analysis in discordant twins	Differentially expressed circulating miRNA associated to PD, taking into account the genetic background/ environmental exposures
miRNomics validation (PG)	qPCR	de novo PD, advanced PD, controls (UMG-GOE) centenarians, controls (UNIBO)	Serum	de novo PD vs controls advanced PD vs controls	Differentially expressed circulating miRNA in early phases of PD not under treatment and in PD under treatment

(*continued on next page*)

Table 2 (*continued*)

Analysis	Techniques	Cohorts	Biospecimens	Comparisons	Scientific questions/Expected results
Metabolomics discovery (CIRMMP)	NMR	PD siblings (UMG-GOE, AUSL-ISNB, SAS)	Serum and urine from UMG-GOE Serum from KI and UNIBO	de novo PD vs centenarians advanced PD vs centenarians controls vs centenarians Association with risk factors in PD siblings	Differentially expressed circulating miRNA associated to PD, ageing and successful ageing Differentially expressed circulating miRNA associated with risk of prodromal PD
		de novo PD, controls (UMG-GOE) centenarians, controls (UNIBO)		de novo PD vs controls de novo PD vs centenarians controls vs centenarians	Metabolic profiles in early phases of PD not under treatment Metabolic profiles associated to PD, ageing and successful ageing
		MZ and DZ twins discordant for PD (KI)		Intra-couple analysis in discordant twins	Metabolic profiles associated to PD, taking into account the genetic background/ environmental exposures
Metabolomics validation (CIRMMP)	NMR	de novo PD, advanced PD, controls (UMG-GOE) advanced PD, controls (SAS) centenarians, centenarians'offspring, controls of different age (UNIBO)	Serum from UMG-GOE and UNIBO Plasma from SAS	de novo PD vs controls Advanced PD vs controls PD (de novo and advanced) vs centenarians'offspring Association with age (controls of different age) and with successful ageing (centenarians)	Metabolic profiles in early phases of PD not under treatment Metabolic profiles in PD under treatment Metabolic profiles associated to PD and not associated to successful ageing Comparison of metabolomic profiles in healthy/successful ageing respect to PD; metabolomic clock (accelerated ageing in PD?)
Proteomics discovery (UCL)	Deep phenotyping by label-free proteomics and nano 2D-LC QTOF MSE	PD siblings (UMG-GOE, AUSL-ISNB, SAS)	Serum	Association with risk factors in PD siblings	Metabolic profiles associated with risk of prodromal PD
		de novo PD, controls (UMG-GOE) centenarians, controls (UNIBO)	Plasma	de novo PD vs controls de novo PD vs centenarians controls vs centenarians	Proteomic profiles in early phases of PD not under treatment Proteomic profiles associated to PD, ageing and successful ageing
		MZ and DZ twins discordant for PD (KI)	Serum from KI	Intra-couple analysis in discordant twins	Proteomic profiles associated to PD, taking into account the genetic background/ environmental exposures
Proteomics validation (UCL)	UPLC-MS/MS targeted proteomics	de novo PD, advanced PD, controls (UMG-GOE) advanced PD, controls (SAS) centenarians, centenarians'offspring, controls of different age (UNIBO)	Plasma and urine from UMG-GOE Plasma from SAS and UNIBO	de novo PD vs controls Advanced PD vs controls PD (de novo and advanced) vs centenarians'offspring Association with age (controls of different age) and with successful ageing (centenarians)	Proteomic profiles in early phases of PD not under treatment Proteomic profiles in PD under treatment Proteomic profiles associated to PD and not associated to successful ageing Comparison of proteomic profiles in healthy/successful ageing respect to PD; proteomic clock (accelerated ageing in PD?)
		PD siblings (UMG-GOE, AUSL-ISNB, SAS)	Plasma	Association with risk factors in PD siblings	Proteomic profiles associated with risk of prodromal PD
Glycomics (UNIBO)	DSA-FACE	de novo PD, controls (UMG-GOE) advanced PD, controls (SAS)	Serum from UMG-GOE Plasma from SAS	de novo PD vs controls Advanced PD vs controls	Glycomic profiles in early phases of PD not under treatment; GlycoAge score Glycomic profiles in PD under treatment
		PD siblings (UMG-GOE, AUSL-ISNB, SAS)	Plasma	Association with risk factors in PD siblings	Glycomic profiles associated with risk of prodromal PD
Metagenomics (AUSL-ISNB)	16S sequencing	PD siblings (UMG-GOE, AUSL-ISNB, SAS)	Stool	Association with risk factors in PD siblings	Metagenomic profiles associated with risk of prodromal PD
Imaging (SAS)	[^{123}I]FP-CIT SPECT T1 3D MRI	de novo PD, controls (UMG-GOE) advanced PD (SAS)	Neuroimaging	de novo PD vs controls de novo PD vs advanced PD Association with other biomarkers in PD	Brain aging in PD using neuroimaging data

Guo et al., 2018; Horvath et al., 2015; Montoliu et al., 2014; Rampelli et al., 2020; Santoro et al., 2018; Sayed et al., 2019). However, centenarians are unavoidably very old people, and it is therefore difficult to disentangle longevity from ageing. For this reason, PROPAG-AGEING envisages the inclusion of centenarians' offspring, a well-established model of healthy ageing, characterized by decelerated ageing (Bucci et al., 2016; Conte et al., 2020; Gentilini et al., 2013, 2012; Horvath et al., 2015; Ostan et al., 2013; Vitale et al., 2012). Centenarians offspring population age range is very similar to those of the major ARDs, including PD, thus it is a priceless instrument to distinguish between healthy versus unhealthy ageing trajectories.

Taken together, these cohorts represent an unprecedented league of datasets and bio-materials to grasp the molecular pathophysiology of PD. All PD patients involved in PROPAG-AGEING have undergone deep phenotyping, including international standards of motor classification (Hoehn and Yahr stages), Unified Parkinson's Disease Rating Scale (MDS-UPDRS) scores, MRI imaging data and the assessment of non-motor symptoms.

4. PROPAG-AGEING design: the envisaged characterizations

In recent years, a growing number of studies has attempted to unveil the molecular basis of PD using omic approaches, offering an in-depth characterization of potentially pathological alterations in specific biological layers like the genome, the epigenome, the transcriptome, the proteome, the metabolome or the metagenome (Redenšek et al., 2018). Results of these studies are usually not performed on the same PD patients, and are not always overlapping nor concordant, possibly because they largely differ in size, type of analyzed samples (genetic or idiopathic PD; de novo or advanced PD patients), type of biospecimen (for example, brain, blood, plasma/serum, stool, urine, CSF) and/or analytical approaches. Most importantly, in the vast majority of cases these studies are disjointed, *i.e.* a certain PD cohort has been characterized using only one or few omicapproaches (Hertel et al., 2019).

PROPAG-AGEING aims at overcoming the fragmentation of data and interpretation inherent in previous studies by characterizing the cohorts described in the previous paragraph using a comprehensive set of advanced omics (whole genome and mtDNA sequencing, RNA-Seq, genome-wide DNA methylation, circulating microRNA, proteomics, metabolomics and glycomics), that whenever possible are applied to the same subjects (Table 2). All the analyses are performed on peripheral biospecimen (whole blood for genetic, epigenetic and transcriptomic analysis; plasma/serum/urine for circulating microRNA, proteomic, metabolomic and glycomic analyses), in accordance with the propagation hypothesis at the basis of PROPAG-AGEING. Furthermore, the use of easily accessible biospecimens allows to identify potential diagnostic and prognostic biomarkers assessable in clinical practice.

The analytical approach envisaged in PROPAG-AGEING allows to identify omic-specific markers, i.e. signatures neatly characterized by a single omic layer, as well as signatures that are better described as "multi-omic" covariates, i.e. "compound signatures" constituted by a cluster of different omics. Several approaches will be applied for the multi-omics integration of PROPAG-AGEING data. One of them consists in the application of multilayer networks (Boccaletti et al., 2014). Multilayer networks have been successfully applied to the integration (and interpretation) of several data types. A multilayer network can be defined as M=(G,C) where G is a set of graphs and C is the interconnection between them. For example in the framework of PROPAG-AGEING project we will implement a multilayer network in which each layer represents a type of omic data. In this way we can both analyse each layer independently (for example analysing communities) and in a fused fashion with the others, obtaining, comparing and evaluating single omic signatures, as well as integrated ones.

In parallel to the multilayer networks approach, we will also use an integration of omics data to be used as input features in a deep learning framework. In particular, we will design experiments to estimate biological age using a deep learning approach. The biological age estimation will be first performed in each omic independently as well as in the imaging data (MRI brain scans available for the cohort). Subsequently, omics will be combined and the integrated dataset will be used in order to undercover integrated and combined effects of omic and imaging integration towards biological age estimation. Furthermore, the available epidemiological, lifestyle and clinical data can be incorporated with omic results and newly generated biochemical data, providing a comprehensive *systems biology* view of the disease.

Given the conceptual assumptions at the basis of PROPAG-AGEING, particular attention is placed to the study of biomarkers of ageing and of inflammageing in the PD cohort. Ageing biomarkers can have different natures and are predictive not only of the chronological age of individuals, but also of their biological age, i.e. a proxy of their healthy status (Cole et al., 2019; Jylhävä et al., 2017). Biomarkers of ageing can therefore be informative regarding age acceleration processes associated to the onset or the progression of PD. The epigenetic clock, a predictor of age based on DNA methylation data, showed an accelerated ageing in whole blood from advanced PD patients respect to controls (Horvath and Ritz, 2015), supporting the role of ageing in PD. Within PROPAG-AGEING, besides epigenetic clocks (Bell et al., 2019), we will consider GlycoAge (Vanhooren et al., 2008), based on glycomic signatures, and brainAge (Cole and Franke, 2017), based on brain imaging data. We will also refer to recently published papers that tracked metabolomic and proteomic changes with age (Johnson et al., 2020; Robinson et al., 2020). Furthermore, omics data will be specifically interrogated in order to evaluate inflammageing, considering pro- and anti-inflammatory molecules in the available datasets, like proteomic data (for example CRP, IL6, TGF beta, TNF alpha, IL10 and IL18) or miRNA data (for example miR-21, miR-155, miR-146).

Importantly, other national and international studies (*in primis* the Parkinson's Progression Marker Initiative – PPMI) are designed to establish a comprehensive set of clinical, imaging and bio-sample data (characterized by a multi-omic approach) that can be used to define biomarkers of PD onset and progression. PROPAG-AGEING originally contributes to this cooperative network of research studies, because the systems biology approach for the analysis of PD and healthy samples is contextualized in the framework of the ageing process.

5. PROPAG-AGEING design: the discovery and the validation phases

To achieve an optimal balance between in depth characterization of the samples and cost-effectiveness of the analyses, PROPAG-AGEING envisages two main blocks of activities:

i) A DISCOVERY PHASE, where a limited number of highly informative samples from the existing cohorts (*de novo* PD patients, twins discordant for the disease, healthy subjects of different ages, centenarians and centenarians' offspring as super-controls) are analyzed in depth by the above-described omics;
ii) A VALIDATION PHASE, where a selection of the most informative molecules (genetic variants, epigenetic and transcriptomic signatures, proteins, metabolites and glycomic markers) emerging from the discovery phase and integrated by the environmental and clinical datasets, are tested in larger existing cohorts of PD patients (*de novo* and advanced), healthy and super-healthy subjects. This phase allows therefore the technical validation of potential biomarkers, possibly by high-throughput techniques alternative or complementary to the omic approaches that have been used in the discovery phase. In addition, the validation phase allows to further investigate the relationship between specific molecular alterations and clinical characteristics of PD.

The validation phase takes advantage of two additional models:

1) Dopaminergic neurons (DAn) obtained from human induced pluripotent stem cells (iPSC) deriving from PD patients, controls and super-healthy controls. Appropriate manipulations of this model (in vitro ageing, exposure to stressors related to neuroinflammaging, etc) allow to functionally validate the molecular alterations emerging in the discovery phase and to evaluate the propagation hypothesis of ageing and PD (Mohamed et al., 2019; Ravaioli et al., 2018).
2) a multi-center cohort of siblings of PD patients, not affected by PD at the time of recruitment but possibly showing more risk factors for PD compared to the general population, specifically recruited in the framework of PROPAG-AGEING. Indeed, a genetic (risk) component is present in the sporadic PD, supported by GWAS studies and observational studies reporting an increased risk of PD associated with a family history of the disease (Berg et al., 2015; Delamarre and Meissner, 2017; Kalia and Lang, 2015). Within PROPAG-AGEING, the molecular alterations emerging in the discovery phase are tested in the cohort of high-risk PD

siblings in order to evaluate their potential as early biomarkers of the disease. The PD siblings' cohort is deeply characterized for several clinical parameters, with particular regard for non-motor symptoms that usually precede the motor dysfunction (premotor or prodromal phase of the disease) by more than a decade (Kalia and Lang, 2015). These non-motor symptoms include sleep disorders (Sateia, 2014), in particular REM Sleep Behavior Disorder, olfactory dysfunction, cognitive impairment, constipation and autonomic dysfunction (Kalia and Lang, 2015). Accurate evaluation of these and other parameters allows estimating the risk of developing PD and to correlate it with the levels of biomarkers identified in the framework of PROPAG-AGEING. Multiple aliquots of different biospecimen (whole blood, plasma/serum, urine and stool) are collected from PD siblings. The collection of stool samples is of particular interest given the emerging role of the gut microbiota and of the gut-brain-axis alterations in ageing and neurodegenerative diseases (Baizabal-Carvallo and Alonso-Juarez, 2020; Elfil et al., 2020; Quercia et al., 2014; Santoro et al., 2018).

6. Conclusions

The conceptual assumption of PROPAG-AGEING is that there is a continuum between healthy ageing and neurodegenerative age-related motor disorders. In this framework, the project has the ambitious aim to identify specific cellular and molecular patterns that can deviate the trajectories of healthy ageing towards the development of PD, or alternatively can protect centenarians and their offspring from developing this and other neurodegenerative disorders. These molecular signatures hopefully will represent reliable early markers of PD and new potentially druggable targets.

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