Meeting Report

The Future of Parkinson's Disease Research: A New Paradigm of Human-Specific Investigation Is Necessary… and Possible

Abstract

Parkinson's disease (PD) is a complex neurodegenerative condition with a multifactorial origin. To date, approaches to drug discovery for PD have resulted in symptomatic therapies for the motor manifestations and signs associated with neurodegeneration but have failed to identify preventive or curative therapies. This failure mainly originates from the persistence of major gaps in our understanding of the specific molecular basis of PD initiation and progression. New approach methodologies (NAMs) hold the potential to advance PD research while facilitating a move away from animal-based research. We report a workshop involving NAM experts in the field of PD and neurodegenerative diseases, who discussed and identified a scientific strategy for successful, human-specific PD research. We outline some of the most important human-specific NAMs, along with their main potentials and limitations, and suggest possible ways to overcome the latter. Key recommendations to advance PD research include integrating NAMs while accounting for multiple levels of complexity, from molecular to population level; learning from recent advances in Alzheimer's disease research; increasing the sharing of data; promoting innovative pilot studies on disease pathogenesis; and accessing philanthropic funding to enable studies using novel approaches. Collaborative efforts between different stakeholders including researchers, clinicians and funding agencies are urgently needed to create a scientific roadmap and support a paradigm change towards effective, human-specific research for neurodegenerative diseases without animals, as is already happening in the field of toxicology.

1   Introduction

1.1 Background

Parkinson's disease (PD) remains, some two centuries after its discovery and the beginning of serious biomedical research into it, a considerable global burden. PD is the second most common neurodegenerative disease after Alzheimer's disease (AD) (Lebouvier et al., 2009), and its prevalence and incidence are rising significantly. It is estimated that up to 10 million people suffer from PD worldwide (Ball et al., 2019), and this number could double by 2040 (Orozco et al., 2020). No effective and sustainable treatment has been discovered despite much scientific effort. The main therapeutic intervention, levodopa, was first used clinically more than half a century ago, and its efficacy wears off with time (Mena et al., 2009; Mizuno et al., 2018).

More than 23,000 animal studies of PD were published between 1990 and 2018 (Konnova, 2018). Ten percent of these involved non-human primates (NHPs), predominantly in the preclinical testing of new therapies (Grow et al., 2016). These models, and this research, involving neurotoxin-based induction of PD-like symptoms (using e.g., 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)) and genetic modification techniques to replicate genetic variants linked to PD in human studies, have delivered no significant clinical benefit (Zeiss et al., 2017; Marshall and Willett, 2018a). Instead, few drug candidates that were promising in preclinical research have progressed from Phase I to Phase II clinical trials (Müller, 2010).

There is an increasing awareness that animal models, failing to adequately recapitulate human conditions and to reliably translate to human benefit, contribute to translational failure (Pound, 2020; Pound and Ritskes-Hoitinga, 2018; Ingber, 2020; Leist and Hartung, 2013; Bailey and Balls, 2019; Hartung, 2013). Specific challenges pertaining the use of animals for clinical development for CNS diseases have been documented (Geerts, 2009), and it is widely acknowledged that no animal model mimics complex PD neuropathology, nor accurately replicates clinical symptoms (Potashkin et al., 2011; Marshall and Willett, 2018a).

In non-animal research, the limitations of traditional 2-dimensional (2D) tissue culture approaches have undoubtedly also contributed to this situation (Pamies and Hartung, 2017). However, considerable advances in cell culturing techniques, microengineering, microfluidics, computing power, and their multidisciplinary collaboration have allowed the development of human-specific new approach methodologies (NAMs) that could provide a means to change the course of PD research for the better, and to finally realize a better understanding of PD cause, pathology, mechanisms, and effective, long-lasting therapies.

However, in disease research, NAMs are often viewed as tools to provide information for subsequent "confirmation" in animal models rather than as full replacements. There is no detailed framework for how human-specific methods could be used selectively in integrated and intelligent strategies to better inform PD research, and to replace animal-based methods. Such a framework could also identify remaining gaps so that developers of NAMs can prioritize innovation to fill them.

1.2 Workshop aims

The aim of this workshop, which was coordinated by the Center for Alternatives to Animal Testing (CAAT) and the Center for Contemporary Sciences (CCS) and held May 21, 26 and 27, 2021, was to develop a scheme that outlines the basic research and testing approaches necessary to make PD research a success—elucidating pathological mechanisms, identifying knowledge gaps, discovering druggable targets, and developing new therapies that can enter the drug development pipeline—focusing on human-specific, non-animal methods of research.

While this workshop initially served as an academic exercise, we intend to develop its outcomes towards practical use, and to help expedite much-needed changes in how PD research is funded and conducted. Our scheme would support the increasingly widely appreciated case for an urgent transition away from reliance on using animals and toward the use of human-specific approaches in an integrated strategy based on robust scientific evidence for use by academia and industry. This would also help to overcome the

understandable apprehension and objections of stakeholders that are delaying the paradigm shift toward directly relevant, human-specific research and serve as a starting point to give them the confidence to instigate that transition and provide impetus to its continued development.

2 Parkinson's disease

PD is a complex, age-associated, neurodegenerative condition associated with dopamine loss and both motor and non-motor abnormalities. PD is often characterized by the abnormal accumulation and aggregation of alpha-synuclein (αSyn) in the form of inclusions called Lewy bodies (LB), and Lewy neurites (LN). Several genetic and environmental factors influence PD risk, with distinct factors prevailing in diverse patients. These factors congregate in specific pathways, involving mitochondrial dysfunction, protein aggregation, oxidative stress, defective autophagy, and neuroinflammation (Simon et al., 2020; Balestrino and Schapira, 2020).

During the workshop, the participants discussed what is really known about human PD, as much of the identified knowledge comes from animal models with questionable translation to the human disease. It is important to define where we are now, and where we need to go (Brás et al., 2020; Oliveira et al., 2021).

2.1 Where does Parkinson's disease start and what are the cell types and organs of importance?

Although the degeneration of dopaminergic neurons in the substantia nigra (SN) is thought to play a key role in the development of PD, emerging evidence suggests that the pathological process does not start in the SN, but more likely elsewhere in the nervous system, in the peripheral autonomic nervous system, or even in other organs, such as the gut (Adams et al., 2019; Reynolds et al., 2019; Scheperjans et al., 2018). αSyn aggregates have been found in the gut, and they are highly abundant in blood (erythrocytes and platelets) and in other peripheral tissues (Abd Elhadi et al., 2019; Pediaditakis et al., 2021). It has also been suggested that gut microbiota may be involved in PD pathogenesis (Keshavarzian et al., 2020).

During the prodromic stage, non-motor symptoms appear to precede motor deficits (Borghammer and Van Den Berge, 2019; Gaig and Tolosa, 2009). At the stage of motor symptoms presentation, more than 80 percent of dopaminergic neurons have already died (Masato et al., 2019). An important aspect that emerged during the workshop discussion was that PD researchers often focus on the study of dopaminergic neurons and SN while ignoring the potential role of other tissues in the development of PD.

2.2 Neurodevelopmental versus neurodegenerative disorder

The hypothesis that PD is a neurodevelopmental disorder that is compensated for a long time before becoming symptomatic is under discussion. A developmental defect, the "first hit", might not cause the disease immediately but may increase the susceptibility for disease onset following a "second hit." Even a small developmental defect, like a delay or acceleration in dopaminergic neuron identity specification, could result in a decreased compensatory function or lowered fault tolerance of the system. In the event of a pre-existing situation of imbalance, one or more further hits could disrupt the system sufficiently to disbalance it, leading to PD (Schwamborn, 2018). This is also consistent with a possible role for αSyn during development (Morato Torres et al., 2020).

2.3 Mechanisms

The main obstacle to developing effective disease-modifying cures for PD is a limited understanding of the crucial molecular mechanisms that elicit neurodegeneration. Genetics accounts for only 10 to 15 percent of PD cases (Day and Mullin, 2021). Routinely observed mutations in both familial and idiopathic PD involve several genes including *LRRK2, PRKN, and aSyn (SNCA)*.

αSyn aggregation is a common mechanism in neurodegeneration, but it is not essential to the pathological process. Indeed, PD is also observed without αSyn aggregation, and vice versa, i.e., incidental Lewy body disease has been observed upon autopsy in individuals without clinical signs of parkinsonism or dementia. More than 20 years after the identification of the protein components of these inclusions, and more than one hundred years after their first identification, we still cannot pinpoint LB as the cause of PD. We still do not know the mechanisms by which αSyn becomes toxic and how it causes synucleinopathies. A hypothesis could be that posttranslational modifications, determining localization and three-dimensional folding, are related to critical changes in protein functions. There is increasing evidence suggesting that aberrant αSyn can spread from neuron to neuron and initiate aggregation in different brain regions as seeds in a prion-like manner (Brás et al., 2020; Abounit et al., 2016; Ma et al., 2019). Identifying an αSyn transfer mechanism could suggest strategies to target the protein and prevent its spread, thereby preventing disease progression.

3 Current new approach methodologies in Parkinson's disease

The workshop experts presented and discussed their own research using NAMs. Promising NAM models that are relevant for PD research include: 1) 2D and 3D human-relevant models, based on the use of human-cell lines or patient-derived cells, including induced pluripotent stem cells (iPSCs) and iPSC-derived organoids; and 2) advanced bioengineered *in vitro* platforms (e.g., organs-on-a-chip). Promising methods include 1) multiple "omics" analysis (e.g., genomics, proteomics, transcriptomics, metabolomics) resulting from investigations of patient-derived biological samples through high-performance analytical approaches and databases; 2) advanced imaging techniques, and 3) computational models. A list of main currently available NAM models and methods, their possible applications to PD research, limitations, and potential ways to overcome them, are summarized in Table 1.

Tab. 1: Examples of currently available NAMs, their possible applications to PD research, their limitations, and potential ways to overcome these limitations

NAMs	Examples of applications to PD research	Limitations	Possible ways to overcome limitations
LUHMES cell models	Studying basic biomolecular pathogenetic mechanisms High-throughput screening of candidate neuroprotective drugs	They might not be fully representative of the complex physiology of the brain and/or of PD pathophysiology.	Co-culturing with other cell types Culturing in 3D context
		Continual synthesis and release of dopamine in culture	Increasing work to optimize culture protocols
		Difficult to culture for extended periods of time	Culturing in 3D context
iPSC-derived brain organoids	Studying developmental components of PD Investigating PD-relevant pathogenetic mechanisms Deciphering risk factors Exploring genetic and sex differences, gene-environment interaction Identifying small molecule drugs to rescue PD pathology High-throughput screening for compounds that can inhibit or ameliorate PD pathogenesis Personalized medicine	Generating high-quality iPSCs is expensive and time-consuming.	Cost will likely decrease over time PD-iPSC lines commercially available Generating more characterized and sophisticated models of brain organoids
		Organoids represent an early stage of embryonic development while PD is a late-onset disease.	Inducing an overexpression of aging-related genes (such as progerin) to recapitulate aging and late-onset diseases Direct conversion of aging donors' fibroblasts into neurons to help retain aging-related transcriptional signatures
		Cerebral organoids cannot recapitulate disease progression.	Combine with patients' *in vivo* and postmortem studies
		Lack of adequate oxygen and nutrient supply and the accumulation of metabolic waste	Adding vasculature Combining with fluidic technologies
		Random and uncontrolled nature of organoid growth	Better characterization of models and standardization of culture protocols
Organ-chips, MPS (e.g., brain-chip, substantia nigra brain-chip, neurovascular unit-on-a-chip, gut-brain-liver-on-a-chip, etc.)	To investigate interaction between organs or organ systems relevant to PD, including brain, BBB, intestine, and the gut-brain axis To model the effects of exposure to abnormal αSyn aggregates Performing more physiologically relevant *in vitro* tests for novel therapies; looking at the influence of the gut microbiota iPSC-based OoCs: studying genetic, ethnic, sex differences, and gene-environment interactions Applications for personalized medicine Real time monitoring of electrical activity, and physicochemical parameters	Lack of robust and reproducible iPSC differentiation protocols for derivation of several cell types of the CNS Limited lifespan of certain types of cells, e.g., endothelial cells	Extensive characterization of OoCs and multi-OoCs
Omics/ Multi-omics approaches (e.g., genomics, transcriptomics, proteomics, metabolomics, exposomics)	To define the molecular mechanisms underlying PD pathogenesis Finding biomarkers for clinical detection, progression and treatment Defining genetic and	Integration of different omics datasets is a challenging task that relies heavily on data-mining and machine learning algorithms.	Opportunity to establish committed consortia with a multi-disciplinary approach (e.g., combining molecular biology and bioinformatics expertise (Krassowski et al., 2020).
		Variable reliability levels of heterogeneous data	

	environmental risk factors Stratifying patients' population; to assess signaling pathways		
Advanced imaging	Assessing dopaminergic neuron degeneration before symptom onset To test therapies that could prevent or halt PD before irreversible symptoms Identifying patients who are on trajectory to develop disease for early intervention	High costs Sometimes weak correlations between measures and clinical manifestations	Considering large-scale studies to improve correlations between imaging measurements and clinical manifestations
Computational models (e.g., PBPK, PDPK, QSP)	PBPK: to define kinetics and dynamics of compound exposure at the site of action QSP; to predict long-term effects of drugs; to assess therapeutic potential of novel compounds taking into account individual patient characteristics such as patient-specific genotypes, concomitant medications, and disease conditions on functional clinical scales	Results based only on existing knowledge and input data Performance impaired by low-quality data	Updating models using novel, emerging, human-relevant clinical and preclinical data

3.1 Human cell lines

A better understanding of the molecular and cellular mechanisms underlying PD pathogenesis and progression is essential to developing an effective disease-modifying therapy. Since primary human neural cells, including dopaminergic neurons, are difficult to obtain and maintain in culture, *in vitro* PD research has been mostly carried-out with permanently established cell lines to date (Falkenburger et al., 2016). Although cell lines obtained from cancerous or *in vitro* transformed cells, as well as primary neurons isolated from pre- or post-natal rodents, have been useful in the *in vitro* study of basic mechanisms of PD, they are unlikely to recapitulate all the properties of human neurons *in vivo* (Napoli and Obeid, 2016; Rich et al., 2020; Harischandra et al., 2020).

Thus, cells with high physiological relevance, good batch-to-batch consistency, and large potential culture scale can provide a human-focused, effective, high-throughput screening model for PD. Conditionally immortalized cells, such as the Lund human mesencephalic (LUHMES) cell line, may offer such possibilities (Fig. 1). LUHMES cells are human embryonic neuronal precursor cells that can be maintained as proliferating cells and can be differentiated into morphologically and biochemically mature dopaminergic-like neurons (Schildknecht et al., 2009; Scholz et al., 2011). They are increasingly used to study the biology of dopaminergic neurons and PD *in vitro* (Schildknecht et al., 2009; Harischandra et al., 2020; Schildknecht et al., 2015; Loser et al., 2021; Schildknecht et al., 2013b; Gerding et al., 2019). Differentiated LUHMES cells show spontaneous electrical activities and are able to produce/uptake dopamine like human dopaminergic neurons (Zhang et al., 2014).

Coculture of LUHMES cells with astrocytes has elucidated biochemical pathways for the activation of the pro-toxicant MPTP, a pesticide that causes PD-like symptoms by inducing degeneration of nigrostriatal dopaminergic neurons, to toxicant 1-methyl-4-phenyl-pyridinium (MPP+) (Schildknecht et al., 2017; Schildknecht et al., 2015). Known neuroprotective compounds led to different pharmacological profiles in monocultures and co-cultures, suggesting that the presence of glial cells (astrocytes and/or microglia) and the local production of the toxic metabolite MPP+ within the layered cultures play an important role in neuroprotection (Efremova et al., 2015).
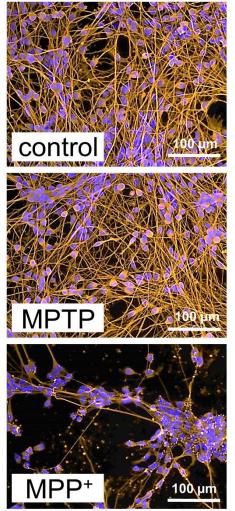
LUHMES cell models can be used for investigating αSyn and its role in the development of PD (Paiva et al., 2017; Schildknecht et al., 2013a; Schildknecht et al., 2009). They have also been used to study the effects of oxidative modifications (nitration) on the aggregation of αSyn *in vitro* as well as its binding to biological membranes. It has been shown that nitrated αSyn stimulates the aggregation of unmodified αSyn molecules by acting as a catalyst for the process (Schildknecht et al., 2013a).

Adverse outcome pathways (AOPs) collate and organize existing information connecting the modulation of a molecular target (molecular initiating event) through a sequence of crucial biological key events to an adverse outcome. LUHMES models have been used to build an (AOP), showing mechanistic plausibility for epidemiological evidence on the relationship between MPTP exposure via binding to mitochondrial complex I with the onset of parkinsonian motor deficits (Terron et al., 2018).

The LUHMES model system is a closer approximation of human nigrostriatal neurons than traditional cultures and allows the investigation of the complex biochemical pathways underlying PD development as well as the screening of candidate neuroprotective drugs, promising increased human-relevance and translational success. The ease of culturing these cells allows for the setup of cell-based assays for high-throughput screening. An important challenge to overcome is the continual synthesis and release of dopamine in culture (Schildknecht, *unpublished*). The cells are difficult to culture for extended periods of time, but this problem can be overcome by culturing them in 3D neuronal spheres, which allows adequate oxygen and nutrient supply for survival of the innermost cells and small molecules penetration. This model could be suitable to identify environmental contributions to PD pathogenesis as well as to address the processes of resilience and recovery in neurotoxicology and PD in future studies (Smirnova et al., 2016).

Fig. 1: Human dopaminergic neurons (LUHMES)
Human dopaminergic neurons (LUHMES) were differentiated for 6 days. The cells were treated with the protoxicant MPTP of with the active toxicant MPP$^+$ [5 µM] for 48 h. For visualization of cell morphology, cells were stained with an anti-β-III-tubulin antibody (orange), nuclei are stained in blue. Image provided by Stefan Schildknecht.

3.2 Brain organoids

A brain organoid is an artificially grown, *in vitro* miniature organ resembling parts of the brain or defined brain regions (e.g., midbrain organoids) (Fig. 2). Brain organoids are generated by culturing induced pluripotent stem cells (iPSCs), embryonic stem cells, progenitor cells, or multiple types of differentiated cells using specific conditions and media. Organoid brain models allow the simulation of specific changes in neurological disorders and offer several benefits over traditional two-dimensional cultures and whole animal-based approaches in studying PD. The 3D structure increases model complexity over monolayer cultures by allowing more physiologically relevant cell-to-cell interactions and allows cultures to be maintained for longer periods of time. These models are electrophysiologically active, support investigations of increasing complexity, and may be composed of the major cell types in the brain, including neurons, astrocytes, and oligodendrocytes (Smits and Schwamborn, 2020). Human iPSC-derived midbrain organoids are able to produce neuromelanin inclusions that resemble those isolated from the human substantia nigra, in contrast to *in vivo* rodent models, human midbrain dopaminergic neurons generated using traditional 2D cell culture methods, or midbrain-like organoids generated from mouse embryonic stem cells, which typically do not produce neuromelanin (Jo et al., 2016). Midbrain organoid models are being employed for understanding mechanisms of PD, to decipher risk factors, as well as to identify small molecule drugs to rescue PD pathology (Zanetti et al., 2021; Smits and Schwamborn, 2020).

In addition to their derivation from the species of interest, iPSC-based organoid models can be developed using cells from patients with PD. This strategy enables investigation into genetic differences, sex differences, gene-environment interactions, personalized medicine, and clinical transplantation therapy (Schwamborn, 2018).

Since *in vitro* organoid differentiation recapitulates the early stages of brain development, brain organoids may be useful in the study of developmental components of PD and the identification of early biomarkers. If the developmental theory and the "multiple hit" concept are correct, an early identification of the deregulated developmental processes may allow intervention therapy in at-risk individuals before symptoms appear.

Human midbrain-specific organoids derived from PD patients carrying a specific LRRK2 mutation were used to investigate PD-relevant pathogenetic mechanisms (Smits et al., 2019). These organoids recapitulated key characteristics of the disease, including decreased amounts of midbrain dopaminergic neurons compared to isogenic control organoids. This pinpoints an impaired midbrain dopaminergic neuron specification during organoid development, supporting the hypothesis that PD has a neurodevelopmental component (Smits et al., 2019). Further studies support the usability of midbrain organoids for PD *in vitro* disease modeling (Kwak et al., 2020; Nickels et al., 2020; Mohamed et al., 2021; Kim et al., 2019; Zanetti et al., 2021; Monzel et al., 2020; Jo et al., 2021).
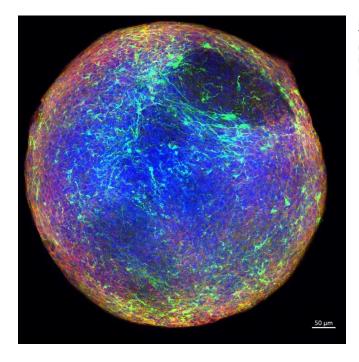
Fig. 2: 30-day old midbrain organoid
The neuronal marker MAP2 is shown in red, the dopaminergic neuron marker TH is shown in green, and the DNA in cell nuclei is shown by Hoechst staining in blue. Image provided by Mudiwa Nathasia Muwanigwa (University of Luxembourg).

iPSCs differentiated from parkin (PRKN)-mutated patients into midbrain organoids confirmed decreased numbers of astrocytes in PRKN-mutated organoids compared with age- and sex-matched controls. This correlates with observations in human brains from individuals with PRKN mutations compared with subjects with idiopathic PD or healthy individuals and suggests the probability of an astrocyte-associated cell death mechanism for dopaminergic neurons in the brains of PRKN-mutated patients (Kano et al., 2020).

Inducing an overexpression of aging-related genes (such as progerin) in iPSC-derived organoids is a technique that has been applied as an artificial aging system to recapitulate aging and late-onset diseases like PD (Hu et al., 2018; Miller et al., 2013). Alternatively, the direct transdifferentiation of aging donors' fibroblasts into neurons (namely induced neurons, or iNs), circumventing cell reprogramming toward an embryonic phenotype, has shown promise: iNs were found to retain aging-related transcriptional signatures when compared to iPSCs and their neuronal derivatives (Mertens et al., 2015).

Miniature brain organoids also allow the characterization of cellular responses through electrophysiology, immunocytochemistry, histology, flow cytometry, and gene expression, and to associate phenotypic changes to a drug's mechanism of action more rapidly and accurately than ever before. Certain types of brain organoids allow human-relevant oligodendrocyte myelination. Examples of commercially available 3D miniature brain organoids are BrainSim®[1] by AxoSim, which are composed of neurons, astrocytes, and oligodendrocytes, and human midbrain-organoids by OrganoTherapeutics[2]. Functional activity of neural organoids can be measured using commercially available MEA systems such as by Axion Biosystems[3].

Limitations of organoid models include the lack of a vasculature and the random and uncontrolled nature of their growth. Future studies will involve generating better characterized and more sophisticated models of brain organoids, including diverse brain regions with immune cells and vasculature, as well as their combination with additional organ-specific organoids such as the gut, which may also allow the study of the impact of gut microbiota on disease development (Reiner et al., 2021). These models will allow the study more extended patient cohorts and the evaluation of potential therapeutics.

3.3 Organ-chips

Organ-chips are multi-channel 3D microfluidic cell culture devices that model the physiology, tissue-tissue interfaces, and microenvironment of functional units within human organs, as well as inter-organ communication (Bhatia and Ingber, 2014). Organ-chips are made of transparent flexible polymer, the size of a memory-stick, typically containing two fluidic microchannels separated by a porous flexible membrane that can be coated with extracellular matrix proteins and seeded with cells on either side. Media flow can be precisely controlled, providing nutrients as well as the ability to collect the effluent for analysis (e.g., proteomics or metabolomics). The fluidic channels may be positioned between two vacuum chambers, which can be used to control cyclic stretch in organ-chips such as a gut-on-a-chip to recreate the mechanical forces cells experience inside the body during peristalsis (Kasendra et al., 2018). Organ-chips can be used to investigate interactions between organs or organ systems relevant to PD, including brain, blood-brain-barrier (BBB), intestine, and the gut-brain axis.

Pediaditakis et al. (2021) used Emulate organ-on-a-chip technology to develop a human brain-chip representative of the substantia nigra region of the midbrain comprising iPSC-derived dopaminergic neurons, immortalized microglia, primary astrocytes, primary pericytes, and iPSC-derived microvascular brain endothelial cells, recreating a neurovascular unit (Fig. 3). To model the effects of exposure to abnormal αSyn aggregates and assess the capability of the substantia-nigra brain-chip to generate clinically relevant

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[1] https://axosim.com/brainsim/

[2] http://organo-therapeutics.com/

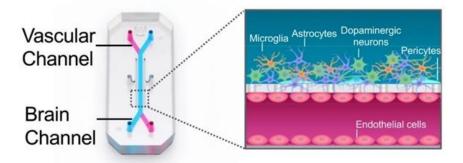[3] https://www.axionbiosystems.com/applications/neural-activity/neural-organoids

Fig. 3: Substantia nigra brain-chip
Schematic representation of the substantia nigra brain-chip with brain endothelial cells cultured on all surfaces of the lower vascular channel, and dopaminergic neurons, astrocytes, microglia, and pericytes in the upper brain channel, on the upper surface of the central horizontal membrane (Image from Pediaditakis et al., 2021 / CC-BY 4.0).

endpoints, a model of synucleinopathy was built by adding exogenous human αSyn fibrils into the brain channel. This model was able to replicate many crucial aspects of PD, including accumulation of phosphorylated αSyn, uptake of αSyn fibrils by neurons, astrocytes and microglial cells, mitochondrial dysfunction, reactive oxygen species (ROS) production, neuroinflammation, neuronal cells death, and impaired BBB function. Interestingly, transcriptomic analysis of the endothelial cells in the brain-chip revealed the upregulation of genes involved in inflammation, oxidative stress, autophagy, efflux system, and extracellular matrix deposition and the downregulation of genes that encode for tight junction proteins, suggesting a new pathogenic mechanism and a potential target for therapeutic intervention in PD. Trehalose-treated brain-chips showed reduced neuroinflammation and BBB permeability, rescuing the damaged tight junctions following exposure to αSyn fibrils (Pediaditakis et al., 2021). In summary, the brain-chip provides a promising model for the study of the specific disease mechanisms, including the dynamics of BBB dysfunction and may be useful to characterize the response to PD therapies and identify and evaluate associated biomarkers of the disease.

Nerve-on-a-chip platforms of iPSC- or primary culture-derived 3D organoids allow nerve conduction analyses to measure changes in electrophysiological properties in response to stimuli. Examples of commercially available organs-on-a-chip relevant to PD research are the NerveSim® platform[4] by Axosim and BBB/Brain-on-a-chip by EmulateBio[5] and by ElveFlow[6] (Elveflow 2022).

3.4 "Omics" approaches

High-throughput technologies, such as genomics, transcriptomics, proteomics, and metabolomics, are currently applied to define the molecular mechanisms underlying PD pathogenesis (Redenšek et al., 2018).

With regard to genomics, the most recent large-scale genome-wide association studies (GWAS) have discovered more than 90 common variants for sporadic PD risk, age at onset and progression across more than 80 genomic regions (Blauwendraat et al., 2019; Foo et al., 2020; Iwaki et al., 2019; Nalls et al., 2019). Recently, a PD GWAS browser tool[7] was created to support the PD research community with the prioritization of genes for follow-up functional studies to detect potential therapeutic targets (Grenn et al., 2020).

Transcriptomics has the potential to provide substantial insight into disease processes. Transcriptome-wide association studies (TWAS) in bulk human brain tissues have found numerous molecular signatures associated with the disease (Borrageiro et al., 2018). Although these studies have the potential to shed light on PD pathogenesis, they are limited by heterogeneous cell type composition of bulk tissue samples, and RNA post-mortem degradation (Nido et al., 2020). Performing transcriptomics studies on human-relevant *in vitro* models may help overcome some of these issues. For example, a single-cell transcriptomics study on human *in vitro* models allowed the identification of numerous neuronal subtypes with transcriptionally different profiles and sensitivities to stress, showing cell type-specific perturbations in human dopaminergic neurons. These findings have implications for cell replacement therapies (Fernandes et al., 2020).

The role of protein modifications during PD pathogenesis could be better understood by the extensive application of proteomics approaches. Several proteomic analyses performed on brain tissue, cerebrospinal fluid, and blood of PD patients have detected a wide spectrum of protein modifications underlying disease pathogenesis (Dixit et al., 2019; Posavi et al., 2019; Rotunno et al., 2020; Werner et al., 2008). These studies are limited by the types of brain areas or biofluids accessible for research.

A growing amount of research has demonstrated that metabolomics profiling holds great promise in providing insights into molecular pathogenesis and could be very effective in detecting candidate biomarkers for clinical detection, progression and treatment of PD, as well as in the study of the influences of environmental risk factors (Dong et al., 2018; Goldman et al., 2018; Peters et al., 2019; Sinclair et al., 2021; Troisi et al., 2019).

Omics approaches can also be applied to study gut microbiota composition and functions in PD patients. Gut microbiome alterations in PD have been reported frequently (Mao et al., 2021; Romano et al., 2021). A recent study showed that fecal microbiome and metabolome composition in PD was considerably different from controls, supporting a role for microbial metabolites as potential targets for developing new biomarkers and treatments in PD (Tan et al., 2021).

3.5 Advanced imaging techniques

Non-invasive imaging in living patients has rapidly progressed in recent decades. Positron emission tomography (PET) (Loane and Politis, 2011), diffusion tensor MRI (Zhang and Burock, 2020), functional MRI (fMRI) (Baggio and Junqué, 2019), transcranial sonography, magnetoencephalography (Agrawal and Biswas, 2015), proton magnetic resonance spectroscopy (PMRS) (Mazuel et al., 2016), and single photon-emission computed tomography (SPECT) (Pahuja et al., 2020) have all provided valuable insights that can

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[4] https://axosim.com/nervesim/
[5] https://emulatebio.com/brain-chip/
[6] https://www.elveflow.com/microfluidic-reviews/organs-on-chip-3d-cell-culture/microfluidic-brain-on-chip/
[7] https://pdgenetics.shinyapps.io/GWASBrowser/

be followed non-invasively and longitudinally. Furthermore, human connectomics, supported by techniques such as MRI tractography, allows for the reconstruction of 3D neuronal networks, brain anatomy, and PD-related neuroanatomical modifications (De Micco et al., 2021; Krismer and Seppi, 2021; Theisen et al., 2017).

Specific PET tracers allow distinction of PD from overlapping diseases, such as multiple system atrophy, and can also be used as a biomarker for dopaminergic neuron degeneration before symptom onset. This could allow the testing of therapies to prevent or halt PD before irreversible symptoms present (Loane and Politis, 2011). The ability to gather quantitative longitudinal data relative to biomarkers makes non-invasive imaging techniques essential to current and future clinical trials (Saeed et al., 2017). Development of a PET tracer for αSyn is underway (Korat et al., 2021) and promises to be a game changer like it was for amyloid and tau PET tracers in AD. In this context, the recent NIH initiative "Center Without Walls for Imaging Proteinopathies with PET" engages several academic and industrial centers with different areas of expertise to develop imaging biomarkers.

As for many NAMs, new imaging technologies will be required to overcome excessive operational costs. Moreover, investigators will need to increase the precision of the identifying correlations between measures and PD-related clinical manifestations, as well as formulate procedures to deal with complex data sets to improve quantification of imaging features. Despite current limitations, existing imaging technologies represent important tools to assist human-relevant PD research approaches and should be employed on a wide scale in clinical studies to improve correlations between imaging measurements and clinical manifestations.

Further progress in non-invasive human brain imaging could also reduce the reliance on conventional pathological tissue analysis in inadequate animal disease models.

3.6 Computational models

A major and often underestimated challenge is the translation of preclinical neuroprotective findings of putative therapeutics to the human patient. The ideal drug must achieve sufficient target exposure at the site of action, with limited off-target effects, a long enough duration to actively engage the target, and needs to be devoid of toxicity. For compliance reasons, the formulation should ideally be orally once-a-day, although PD patients are generally open to other, more invasive approaches. In addition, any new drug needs to have minimal pharmacokinetic (PK-PK) or pharmacodynamic (PD-PD) interactions with standard-of-care medications in PD patients.

Physiologically based pharmacokinetic (PBPK) and pharmacodynamic (PDPK) modeling is suitable to define the kinetics and dynamics of compound exposure at the site of action and to simulate the interaction of pharmacological agents with transporters at the BBB to derive an estimate of brain or cerebrospinal fluid penetration.

Quantitative systems pharmacology (QSP) is a relatively new approach aiming to predict pharmacodynamic effects and to assess the therapeutic potential of novel compounds, taking individual patient characteristics into account. For PD, this approach is based on mechanistic and biology-informed modeling of the interaction between a compound and neuronal circuits to simulate the pharmacodynamic effects of a drug in conjunction with patient-specific genotypes, concomitant medications, and disease conditions on functional clinical scales (Geerts et al., 2013, 2020). To discover novel targets for PD treatment and potential synergistic pharmacodynamic effects between different therapeutic compounds, Roberts et al. (2016) built a computer-based QSP platform of the closed cortico-striatal-thalamic-cortical basal ganglia loop of the dorsal motor circuit. Results indicate that the platform can achieve a strong correlation with past clinical trials, including therapeutic interventions that did not show clinical benefit (Roberts et al., 2016). This approach allows an estimation of the impact of individual standard-of-care medications, not only on possible biomarkers but also on a clinically relevant functional outcome such as Unified Parkinson's Disease Rating Scale (UPDRS) or off-time. In addition, the platform is patient-centric as it is calibrated using historical clinical trials. Taken together, PBPK and QSP modeling allow for optimal clinical trial design to increase the probability that therapeutic interventions with potential in preclinical assays demonstrate a clinically relevant signal in clinical trials. The main limitation of computational models is that results are based only on existing knowledge and input data, and, consequently, feeding and updating models using novel, emerging, human-relevant data is crucial.

4   Future directions - How can we improve PD research?

Many years of high-cost failures (ethical and financial) in translating research findings and treatment candidates from preclinical (animal) disease models to human benefits/ therapeutic use demand a serious reconsideration of the confidence placed in animal models in PD research. In addition to the failure in drug development associated with the use of inadequate preclinical (both *in vivo* and *in vitro*) models, which have questionable relevance to humans, the use of these models in an excessively reductionist approach to dissect the possible contributions of single gene(s), protein(s) or mechanism(s) to the onset of a complex, multi-factorial human condition such as PD might have contributed to the translational failure. For these reasons, some key issues were highlighted by the workshop participants that should be seriously considered, with the aim to refocus current and future research strategies and priorities in PD research.

4.1 Integration of new approach methodologies to enhance Parkinson's disease research

Human-specific NAMs should be used to investigate, establish, and explain disease processes at multiple levels of biological complexity, from the molecular level to cells/tissues/organs, to organism/population levels (Fig. 4). Combining novel human-based cellular and computational models with non-invasive imaging techniques, epidemiological and clinical data would enable human-relevant data discovery, while moving away from animal-based PD research. This approach would provide multidimensional insight into the association between PD pathogenetic processes and the influence of the environment on them, allowing the acquisition of comprehensive and holistic biological information in a human context.

Merging different organoids to generate complex multiorgan-on-a-chip (multi-OoC) systems has the potential to transform medical research by opening new avenues for understanding multi-organ diseases and for developing personalized treatments (Picollet-D'hahan et al., 2021). For example, a gut-brain axis-on-a-chip (Kim et al., 2021) and a multi-organ-on-a-chip system including brain, liver, and intestine (Trapecar et al., 2021) are promising developments. The strategic incorporation of multi-OoC into physiologically based PBPK/PDPK model-aided drug development (Carney, 2020), and the integration of multiparametric biosensors for real-time
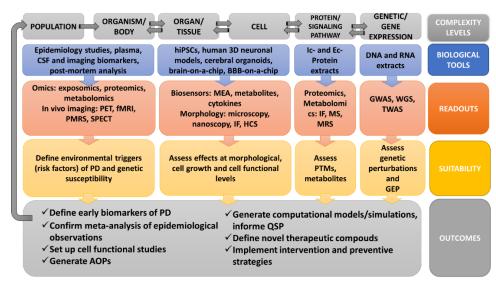
Fig. 4: Overview of novel NAMs, tools and readouts suitable for the design of integrated, human-focused PD research accounting for multiple levels of complexity, from molecular to population level
Abbreviations: AOPs, adverse outcome pathways; CSF, cerebrospinal fluid; fMRI, functional magnetic resonance imaging; GEP, gene expression profiling; GWAS, genome-wide association studies; HCS, high content screening; IF, immunofluorescence; Ic and Ec, intracellular and extracellular; iPSCs, induced pluripotent stem cells; MEA, microelectrode array; MRS, magnetic resonance spectroscopy; MS, mass spectrometry; PET, positron emission tomography; PMRS, proton magnetic resonance spectroscopy; PTMs, post translational modifications; QSP, quantitative systems pharmacology; SPECT, single photon-emission computed tomography; TWAS, transcriptome-wide association studies; WGS, whole genome sequencing.

monitoring of tissue constructs (Ferrari et al., 2020; Liang and Yoon, 2021) holds promise for the advancement of neurodegenerative disease research.

Genetic factors, gene expression, proteins linked with disease or signaling pathways, post-translational modifications, relevant metabolites, and the microbiome can be studied with a wide array of omics approaches, next-generation sequencing technologies, gene expression profiling, mass spectrometry, and magnetic resonance spectroscopy (Fig. 4). These, together with integrated computational modeling, are already laying the groundwork for a systems-biological understanding of disease etiopathology without the use of animals, and thus without the problem of interspecies differences. Moreover, multi-omics approaches may enable the stratification of patients into meaningful disease subgroups, resulting in improved disease management and the development of effective treatments (La Cognata et al., 2021).

Human *ex vivo* materials derived from patients and healthy subjects, such as CSF, plasma and blood samples, post-surgical biopsies, and post-mortem tissues can address population/body biological complexity and be valuable to find early biomarkers. Collecting comprehensive data from both healthy individuals and PD patients is critically important to validate/inform human-based *in vitro* and *in silico* models and to advance PD research. The Michael J. Fox Foundation[8] (MJFF) (Marek Kenneth, 2011) collects clinical, imaging, and genetic data from PD patients and healthy people to provide the research community with critical human-relevant insight into the lived experience, genetics, and variability of PD. Similarly, the ADNI (Alzheimer's Disease Neuroimaging Initiative), has fostered tremendous progress regarding plasma biomarkers and longitudinal personalized trajectory of brain pathology and clinical outcomes of AD over the last 20 years (Veitch et al., 2021).

The application of an analytic approach like the adverse outcome pathways (AOPs) framework (Paini et al., 2021) could help to better categorize existing knowledge about the factors and events associated with PD, to make gaps and overlaps in the knowledge of the disease more evident, and to suggest novel research strategies. The AOP[9] framework allows the integration of many types of information at different levels of biological organization, from molecular to population scale, providing coherent, biologically based theories and hypotheses to predict the outcome of initiating events. The construction of PD related-AOPs has begun (Terron et al., 2018). Intensive efforts to integrate existing PD data to build upon existing AOP efforts could play a substantial role in transforming our ability to identify effective preventative therapies (Marshall and Willett, 2018b). Further PD-associated pathways exist in the Kyoto Encyclopedia of genes and genome database[10].

An outline of the available NAMs, novel tools and readouts available to aid the design of human-oriented PD research, accounting for multiple levels of complexity, is depicted in Figure 4.

4.2 Lessons from Alzheimer's disease research

There was consensus among the experts that we should learn from recent approaches in AD research to inform a PD strategy. More than 400 human trials of potential AD treatments have been conducted but almost no new drugs have reached the market (King, 2018). In AD, one of the major sources of researchers' concern is the poor human relevance of the animal models used in the preclinical stages of drug development and in biomedical research (Pistollato et al., 2016). Moreover, there is an increasing awareness that a paradigm shift toward human-focused, rather than animal-focused research is required to better clarify AD pathogenesis and to enable the design of more effective treatments and preventive interventions (Cavanaugh et al., 2014; Pistollato et al., 2015, 2020).

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[8] https://www.michaeljfox.org/
[9] https://aopwiki.org/
[10] https://www.genome.jp/kegg/

Analogous to the effort in AD research, PD research is now beginning to focus on early modification and prevention of disease, based on CSF or plasma biomarkers that may predict onset of disease before symptoms present (Smedinga et al., 2021; Wu et al., 2011). Identifying patients who are on a trajectory to develop the disease would allow for early interventions that might slow-down or ideally halt the disease process. This strategy shift comes with new challenges that have hindered progress in the field of AD in recent years, where an analogous research strategy led to a number of disappointing trial results. Capitalizing on lessons and experiences from AD research in the field of PD will accelerate progress in PD research (Smedinga et al., 2021). Also, it has become clear that progression in PD may be related to mixed brain pathologies (Buchman et al., 2019).

4.3 Data sharing

For science to work effectively and for society to obtain the full benefits of scientific efforts, it is crucial that scientific data are made freely available. As mentioned above and inspired by the ADNI, the MJFF promotes knowledge-sharing and collates scientific findings on individuals (including *de novo* PD patients, unmedicated PD-, prodromal-, and at-risk patients both with genetic and sporadic PD, and healthy controls). They collaborate with the research community to collect and distribute data resources. The Parkinson's Progression Markers Initiative[11], sponsored by the MJFF[8], is an ongoing longitudinal observational study launched in 2010 that collects comprehensive clinical, imaging, and genetic data, and biological samples, with more than 1,500 participants. This study is supported by a number of industry partners and has built a robust database and biorepository, which is made accessible to the research community to promote biomarker identification and validation as well as therapeutic development. Similarly, the Aligning Science Across Parkinson's[12] (ASAP) initiative is fostering collaboration and resources to better understand the underlying causes of PD by supporting open-access policy. More initiatives like these should be promoted.

Another important fact that emerged during the workshop discussion concerned the importance of sharing and publishing negative data from animal studies. These data need to be accessible to avoid experimental replication, to prevent wasting time and resources, and to ensure that the best, most informed, evidence-based research is funded and conducted.

4.4 Funding and stakeholder engagement

There is a huge need for innovative, novel approaches and trials, including unbiased "fishing expeditions" to find out more about disease pathogenesis. This type of research approach is essentially data-driven or observational research, and its primary objective is to seek meaningful insights, novel mechanisms, patterns or correlations within the data or subject of observation.

The experts mentioned "risk aversion" to novel concepts as a reason for a lack of robust funding to test novel ideas for PD research. This is where philanthropy can play an important role: Philanthropic funding can encourage innovative research by supporting studies that might not otherwise be funded and by promoting a more collaborative and open approach to research.

A scientifically driven transition towards a human-based paradigm in toxicology is taking place, following the roadmap initially traced by the landmark report "Toxicity Testing in the 21st Century: A Vision and a Strategy" published in 2007 by the U.S. National Research Council (NRC, 2007). A similar paradigm shift appears to be necessary in biomedical research, in particular in the field of neurodegenerative diseases, including PD, where the lack of translatability from animal disease models to human patients is particularly evident and, at the same time, the current available human-based models and their future potential applications are very promising. A collaborative effort between different stakeholders including researchers, clinicians, and funding agencies, is urgently needed to create a scientific roadmap and support a paradigm change towards effective, human-specific PD research without animals.

Key recommendations to improve PD research are summarized in Table 2.

Tab. 2: Key recommendations to improve PD research

1	Combining the use of different NAMs and large-scale epidemiological studies accounting for all levels of complexity, from molecular, to cell/tissue/organ, to organism, to population level, while moving away from animal-based research ● Integrating different human-based models and tools (e.g., simpler with more complex models, organoids with fluidic technologies, multi-OoC-platforms, human-on-chip, multi-omics approaches); ● Combining NAMs with large scale epidemiological studies to account for all levels of complexity; ● Leveraging the AOP and PPMI concept to structure existing knowledge about the factors and events concerned with PD
2	Learning from recent approaches in AD research to inform PD strategy ● Identify patients who are on a trajectory to develop disease for early intervention; ● Learn from mistakes: decades of animal-based studies in AD did not result in an effective cure;
3	Sharing data ● Science data should be openly available. Need for robust databases accessible to the research community to promote biomarker identification/validation and therapies development; ● Sharing and publishing negative data on animal studies to avoid experiments' replications making sure that the best, most informed resources are done;
4	Promoting unbiased "fishing expeditions" research to find more about disease pathogenesis;
5	Focusing on philanthropic funding that has the ability to take up the risk and support studies that might not otherwise be funded: Risk aversion to novel concepts often results in a lack of robust funding to test new ideas or PD research.
6	A collaborative effort between different stakeholders including researchers, clinicians, and funding agencies is urgently needed to create a scientific roadmap and support a paradigm change towards effective, human-specific research for neurodegenerative diseases without animals, similar to what is already happening in the field of toxicology.

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[11] https://www.ppmi-info.org/
[12] https://parkinsonsroadmap.org/

5   Conclusion

During the workshop, participants discussed what we really know about Parkinson's disease in humans, as much of the available information comes from animal models, whose relevance to human disease is questionable and has not led to the needed treatment and cure breakthroughs. The experts presented their own research using NAMs, discussing their advantages, limitations, and potentials for PD research. To advance PD research, we must decisively move away from the quest for improved animal models in favor of advanced methods focused on human biology. A better framework for PD research, based on the integration of NAMs at multiple biological scales, would allow an improved understanding of disease mechanisms, better preventive strategies, as well as enabling new treatments to progress 'from bench to bedside' more quickly and cost-effectively. There was a consensus among the experts on the need for a collaborative effort between different stakeholders including researchers, clinicians, and funding agencies, to create a scientific roadmap and support a paradigm change towards effective, human-specific PD research without animals.

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Acknowledgement

Conflict of interest

Hugo Geerts is employee and shareholder of Certara, US. Iosif Pediaditakis is a former employee of Emulate, Inc. and holds equity interests in Emulate, Inc. Jens Christian Schwarmborn is co-founder and shareholder of OrganoTherapeutics.

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