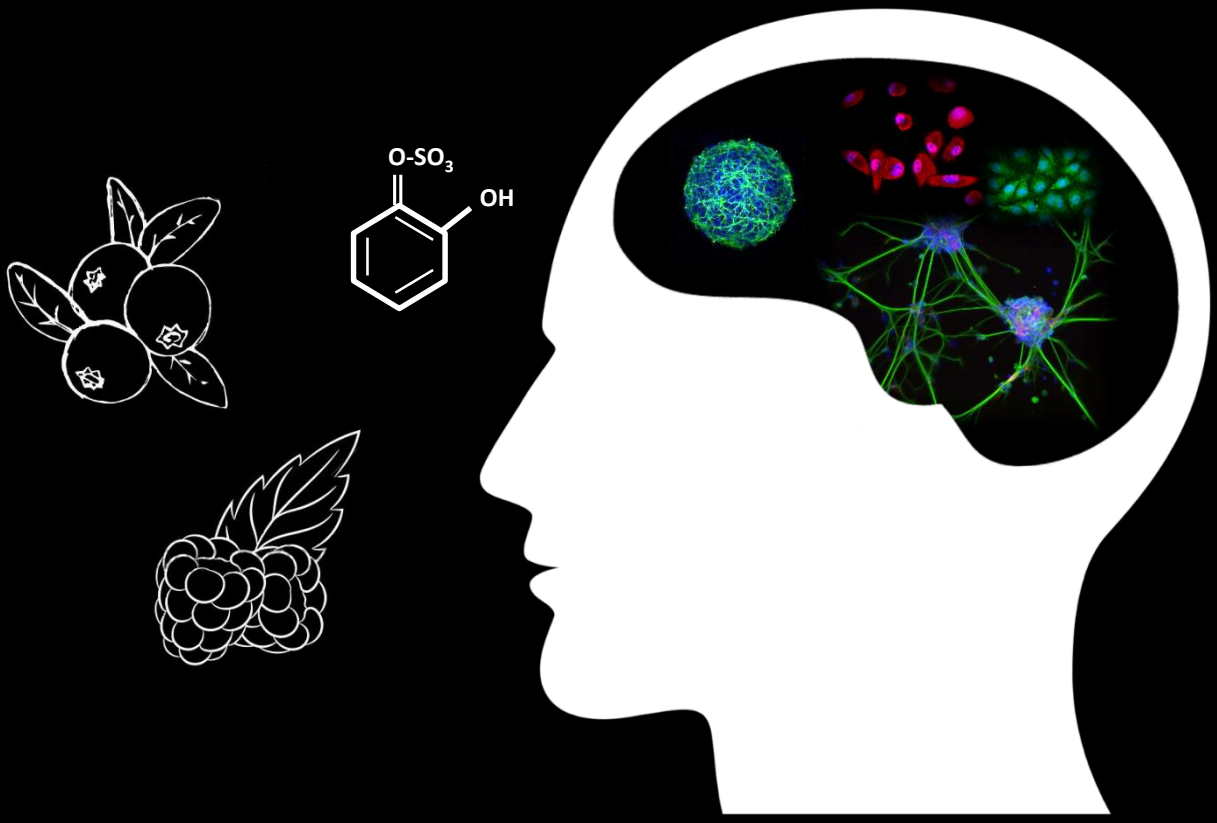
Deciphering the potential of berries polyphenol metabolites for Parkinson's Disease

Digestion, blood-brain barrier transposition and neuroprotective effect

Inês Margarida Lourenço Figueira



Dissertation presented to obtain the Ph.D degree in:

Biochemistry, specialization in Neurosciences

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
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UNIVERSIDADE NOVA DE LISBOA

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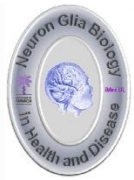

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Abstract

Age-related complications such as neurodegenerative disorders comprise multifactorial disorders which are increasing and remain cureless. The possibility of altering the progression or the development of these multifactorial diseases through diet is an emerging and attractive approach with increasing supporting data. Epidemiological and clinical studies have highlighted the health potential of diets rich in fruits and vegetables. Such food sources are rich in (poly)phenols, natural compounds with increasing associated health benefits, having potential to prevent and/or retard the development of such disorders. In fact, studies with (poly)phenols arising from our diet (dietary (poly)phenols) have been shown their multipotent and pleiotropic ability to modulate several cellular and molecular pathways. The prevention and treatment of neurodegeneration, characterized by a mechanistic complexity, will require novel multi-targeted therapeutic strategies, targeting different disease hallmarks. In that sense, dietary (poly)phenols can emerge as an reliable pleiotropic alternative, with potential to be further explored.

Berries are amongst the most promising rich sources of (poly)phenols with associated brain health benefits, presenting significant implications for neurodegenerative disorders, such as Parkinson's disease (PD). In fact, PD is one of most common age-related neurodegenerative disorders, still without cure, being characterized by several aspects such as dopaminergic cell loss, severe mitochondrial dysfunction, increased oxidative stress and neuroinflammation. The multiplicity of causes/effects that characterize PD raises the need for further investigation for multitarget agents, where berries-derived (poly)phenols can play an important role.

Berries (poly)phenols' potential against chronic oxidative stress was already described to be potentiated upon simulated gastrointestinal digestion, being the protective potential of the bioaccessible components derived from *in vitro* digestion (BDP) higher than the original extract and going beyond the direct radical scavenging properties of parent (poly)phenols. Modifications along digestive process, comprising absorption, distribution, metabolism and excretion, as well as the potential to overcome organs and tissues barriers should be better understood to more accurately design nutritional therapies to tackle disease progression. In here, these berries components derived from *in vitro* digestion, have proven to be transported across the endothelial cells of the blood-

brain barrier (BBB), presenting strong neuroprotective effects against major neurodegeneration hallmarks. Associations between their cytoprotective capacity and the underlying molecular mechanisms highlighted mTOR signaling and unfolded protein response mechanisms as top canonical pathways regulated by BDP. However, to be effectively neuroprotective, these berries components must be not only bioaccessibe but also bioavailable.

In fact, berries-derived bioavailable (poly)phenol metabolites were detected in urine and plasma samples from human individuals and, from a set of metabolites identified and correspondent circulating concentrations, simple phenolic sulfates emerged as the most abundant metabolites. The potential of these known bioavailable phenolic sulfates, arising from colonic metabolism of berries, however, was never assessed before. In this thesis, i*n silico* predictions and *in vitro* transport studies across BBB endothelial cells, at circulating concentrations, provided evidence for their differential transport, likely related to their chemical structure. Moreover, endothelial metabolism of these phenolic sulfates produced a plethora of novel chemical entities with further potential bioactivities. Pre-conditioning with phenolic sulfates improved cellular responses to oxidative, excitotoxicity and inflammatory injuries in different cellular models with increased complexity. Additionally, the role of these metabolites in the attenuation of neuro-inflammatory processes was highlighted: phenolic sulfate anti-neuroinflammatory potential led to a dramatic decrease in TNF-α release which could be related with a regulation of NF-κB nuclear translocation and IκBα levels modulation.

In the end, the potential protective effects of these selected phenolic sulfates in a superior human cell model of PD is investigated. Lund human mesencephalic neural progenitor cells (LUHMES) differentiated in a 3D were used for the superior model of PD, based in 1-methyl-4-phenylpyridinium (MPP^+) lesion of the differentiated neurospheroids. In this PD-like model, bioavailable (poly)phenol metabolites have shown a pre-conditioning protective effect at circulating concentrations. Moreover, such pre-conditioning effect could be related with their proven effects to modulate central pathways such as Nrf2 and p53 signaling in LUHMES neurospheroids. These (poly)phenol metabolites shown to be pleiotropic and modulate central pathways and

key regulators involved in neuroprotection, emerging as a potential new candidates for PD.

In conclusion, a better understanding of cellular and molecular mechanisms by which these small molecules, derived from dietary (poly)phenols, can reach the brain and influence pathological events related with major neurodegenerative diseases was explored. This work is just a small tip of the iceberg though it opens doors for further studies with these phenolic sulfates, perhaps the true preventive effectors emerging from (poly)phenol-rich diets against neurodegenerative diseases.

Resumo

Complicações associadas ao envelhecimento, tais como as doenças neurodegenerativas, fazem parte de um grupo de patologias multifatoriais, cada vez mais incidentes na população e presentemente sem cura. A possibilidade de alterar a progressão ou o desenvolvimento deste tipo de doenças através da dieta é uma abordagem emergente e atrativa, com cada vez mais suporte experimental. Estudos epidemiológicos e clínicos têm reforçado o potencial para a saúde de dietas ricas em (poli)fenóis, uma classe de compostos naturais abundantes em frutas e vegetais. Os (poli)fenóis são compostos naturais associados a um crescente número de benefícios para a saúde, apresentando potencial de prevenir e/ou atrasar o desenvolvimento deste tipo de doenças. Na verdade, estudos com (poli)fenóis provenientes da dieta têm demonstrado a sua capacidade multipotente e pleiotrópica de modular diferentes vias metabólicas, quer a nível celular quer a nível molecular. A prevenção e tratamento do processo de neurodegeneração, caracterizado por uma complexidade mecanística, irá requerer o uso de novas estratégias terapêuticas, capazes de atuar em diferentes aspetos centrais da patologia. Nesse sentido, os (poli)fenóis provenientes da dieta podem surgir como válidas alternativas pleiotrópicas e multialvo, com potencial para serem explorados neste sentido.

Os pequenos frutos encontram-se entre as fontes de (poli)fenóis mais ricas e promissoras, fortemente associados com benefícios para o cérebro, tendo implicações significativas nas doenças neurodegenerativas, tais como a doença de Parkinson (PD). De facto, a doença de Parkinson é uma das doenças associadas ao envelhecimento mais comum, ainda sem cura. É caracterizada por vários aspetos tais como a perda de células dopaminérgicas, disfunção mitocondrial severa, *stress* oxidativo aumentado e neuro-inflamação. A multiplicidade de causas/efeitos que caracteriza a doença de Parkinson tem impulsionado o desenvolvimento da investigação no sentido de encontrar novos agentes multialvo, onde os (poli)fenóis derivados dos pequenos frutos podem desempenhar um papel importante.

Já foi descrito anteriormente que o potencial benéfico dos (poli)fenóis provenientes dos pequenos frutos contra um *stress* oxidativo crónico é aumentado quando estes são submetidos a uma digestão gastrointestinal simulada. Este potencial protetor dos compostos bioacessíveis derivados da digestão *in vitro* é, na verdade, maior do que o

do extrato original, indo para além das propriedades de captação direta de radicais livres por parte dos (poli)fenóis presentes no extrato original. Modificações que ocorrem nos (poli)fenóis ao longo do processo digestivo, tais como absorção, distribuição, metabolismo, e excreção, bem como a sua capacidade de ultrapassar barreiras de tecidos e órgãos do corpo humano, devem ser exploradas e melhor compreendidas de modo a conseguir, de uma forma mais precisa, desenhar terapias nutricionais capazes de intervir na progressão da doença. Neste trabalho, estes componentes dos pequenos frutos derivados de uma digestão *in vitro* (BDP), provaram ser capazes de serem transportados através das células endoteliais da barreira hematoencefálica (BBB), apresentando efeitos neuro-protetores consideráveis contra mecanismos centrais associados à neurodegeneração. Associações entre a sua capacidade cito-protetora e os mecanismos moleculares subjacentes destacou a via do mTOR e mecanismos de resposta a proteínas com incorreta conformação como as principais vias de sinalização canónicas reguladas pelo BDP. Contudo, para que estes componentes derivados dos pequenos frutos sejam efetivamente neuroprotetores, devem ser não só bioacessíveis como também biodisponíveis.

Metabolitos biodisponíveis de (poli)fenóis de pequenos frutos foram detetados em amostras de urina e plasma de voluntários e, de um conjunto de metabolitos identificados e correspondentes concentrações encontradas em circulação, simples sulfatos fenólicos surgiram como os mais abundantes. O potencial destes sulfatos fenólicos biodisponíveis, os quais são derivados do metabolismo dos pequenos frutos pela microbiota intestinal, nunca foi estudado. Nesta tese, previsões *in silico* e estudos de transporte ao nível da barreira hematoencefálica *in vitro,* utilizando concentrações equivalentes às detetadas em circulação, demonstraram o transporte diferencial destes metabolitos, muito provavelmente estando relacionado com diferenças em termos de estrutura química entre eles. Para além disso, o metabolismo endotelial destes sulfatos fenólicos deu origem a uma variedade de novos metabolitos, com potenciais bioatividades a serem exploradas. O pré-tratamento com os sulfatos fenólicos melhorou as respostas celulares a diferentes danos, tais como *stress* oxidativo, excitotoxicidade e inflamação em diferentes modelos celulares de complexidade crescente. Adicionalmente, o papel destes metabolitos na atenuação de processos neuro-

inflamatórios foi evidenciado: o potencial anti-neuro-inflamatório dos sulfatos fenólicos levou a um decréscimo pronunciado na libertação de TNF-α, potencialmente relacionado com uma regulação da translocação nuclear do NF-κB e modulação dos níveis de IκBα.

Para terminar, os efeitos protetores destes sulfatos fenólicos selecionados foram investigados num modelo celular humano de doença de Parkinson. Células humanas, progenitores celulares neuronais mesencefálicos (LUHMES), foram diferenciadas em 3D e usadas para obtenção de um modelo superior de PD, baseado numa lesão aplicadas nos neuro-esferoides diferenciados com 1-methyl-4-phenylpyridinium (MPP^+). Neste modelo de PD, os metabolitos biodisponíveis de (poli)fenóis demonstraram um efeito protetor através de mecanismos de pré-condicionamento quando usados em concentrações equivalentes às circulantes. Para além disso, este efeito de pré-condicionamento poderá estar relacionado com a modulação de vias de sinalização centrais tais como a via do Nrf2 ou do p53. Estes sulfatos fenólicos demonstraram ainda efeitos pleiotrópicos com capacidade de modular vias centrais e reguladores chave envolvidos na neuroprotecção, emergindo como potenciais novos candidatos na prevenção doença de Parkinson.

Em conclusão, uma melhor compreensão dos mecanismos celulares e moleculares pelos quais estes compostos, derivados do metabolismo de (poli)fenóis provenientes da dieta, podem chegar ao cérebro e influenciar eventos patológicos relacionados com as principais doenças neurodegenerativas, foi aqui explorado. Apesar deste trabalho constituir apenas uma pequena ponta do iceberg, abre portas para futuros estudos com estes sulfatos fenólicos, quem sabe, os verdadeiros agentes ativos na prevenção das doenças neurodegenerativas derivados de dietas ricas em (poli)fenóis.

Thesis Outline

This thesis is organized in five parts: introduction, three chapters of experimental work and a general discussion.

In the introduction section, major topics necessary for a broad comprehension of this thesis is described. Major aspects about neurodegenerative diseases and (poly)phenols importance in the disease prevention are present. State of art of major cellular models used for studying (poly)phenols role in Parkinson's disease (PD) is also described. Emphasis to the knowledge gaps of (poly)phenols potential to act inside the brain was considered such as their ability to cross and/or interact with the blood-brain barrier (BBB). Finally, a thesis rational and major work objectives are concluding the introductory section. Part of this section was included in a review published in *Current Neuropharmacology*.

The experimental work is divided in 3 chapters. The first chapter exploits the potential of components derived from the *in vitro* digestion of blackberry to be transported across the BBB and also validates their previous reported neuroprotective potential taking advantage of superior models of neurodegeneration. Moreover, a microarray data analysis highlighted major canonical pathways modulated by these blackberry components that could be responsible for the neuroprotection observed. The work presented in this chapter was published in *European Journal of Nutrition*.

In the second chapter, human bioavailable polyphenol metabolites found in circulation after the ingestion of berries mixture puree were tested for their potential as neuroprotective compounds against common hallmarks of neurodegenerative diseases. These metabolites proven to be differentially transported across the BBB and brain microvascular endothelial cells metabolize them into new cellular metabolites. The human bioavailable polyphenol metabolites were protective against oxidative stress, glutamate excitotoxicity and neuroinflammation. This chapter was published in *Scientific Reports*.

The third chapter comprises a manuscript in preparation validating the neuroprotective potential observed of the human bioavailable polyphenol metabolites in an advanced and superior model of PD. By using a 3D cell model of the disease, recapitulating better the three-dimensional architecture of a brain environment, we observed a hormetic

potential of the metabolites by the modulation of Nrf2 target genes, with neuroprotective potential against important PD hallmarks.

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

This thesis contains a CD with all figures and tables in high resolution quality (appended).

Abbreviations list

1-MePyr-sulf – 1-*O*-methyl pyrogallol-*O*-sulfate (1-*O*-methyl pyrogallol-2-*O*-sulfate and 1-*O*-methyl pyrogallol-3-*O*-sulfate)

2-MePyr-sulf – 2-*O*-methyl pyrogallol-*O*-sulfate

4-MeCat-sulf – 4-methyl catechol-*O*-sulfate (4-methyl catechol-1-*O*-sulfate and 4-methyl catechol-2-*O*-sulfate)

4-MeGA – 4-*O*-methyl gallic acid

4-MeGA-sulf – 4-methyl gallic acid-3-*O*-sulfate

6-OHDA – 6-hydroxydopamine

AADC – aromatic L-amino acid decarboxylase

ABC – ATP-binding cassette

accptHB – number of acceptor hydrogen bonds (QikProp)

ACh – acetylcholine

AChE – acetylcholinesterase

ACN – acetonitrile

ACOX1 – peroxisomal acyl-coenzyme A oxidase 1

ACTB – beta-actin

AD – Alzheimer's disease

ADME – absorption, distribution, metabolism, and excretion

AKAP12 – A-kinase anchor protein 12

Akt (AKT1) – serine/threonine kinase (also called protein kinase B)

ALB – albumin

ALS – amyotrophic lateral sclerosis

AMP – adenosine monophosphate

AMPK – 5' AMP-activated protein kinase

ANGPT2 – angiopoietin 2

ANXA4 - annexin A4

AP – aminopeptidase

AP-1 – activator protein 1

APP – amyloid precursor protein

Arc/Arg3.1 – activity-regulated cytoskeleton-associated protein

ARE – antioxidant response elements

ARS – arylsulfatase

ASK1 (MAP3K5) – apoptosis signal-regulating kinase 1

ASNS – asparagine synthetase

ATCC – American type culture collection

ATF4 – activating transcription factor 4

ATF5 – activating transcription factor 5

Aβ – Amyloid beta

B2M – β2 microglobulin

BACE1 – beta-secretase 1

Bax (BAX) – Bcl-2-associated X protein,

BBB – blood-brain barrier

Bcl-2 (BCL2) – B-cell lymphoma 2

BCL2L1 – BCL2-like 1

BCRP (ABCG2) – breast cancer resistant protein

BDNF – brain-derived neurotrophic factor

BDP – blackberry-driven (poly)phenols

BMEC – brain microvascular endothelial cells

C/EBP – CCAAT/enhancer-binding proteins

CALR – calreticulin

cAMP – cyclic adenosine monophosphate

Cat-sulf – catechol-*O*-sulfate

CBG – cytosolic β-glucosidase

CCND1 – cyclin D1

CCND2 –cyclin D2

CD40 – cluster of differentiation 40

CDC45 – cell division cycle 45

CDC6 – cell division cycle 6

CDF – chip definition file

CDKN1A – cyclin dependent kinase inhibitor 1A

CDKN1B – cyclin dependent kinase inhibitor 1B

CEBPB – CCAAT/enhancer-binding protein beta

CEBPG – CCAAT/enhancer-binding protein gamma

C_{max} – maximum (or peak) serum concentration

CNS – central nervous system

COMT – catechol O-methyl transferase

COX-2 – cyclooxygenase-2

c-Raf (RAF1) – RAF proto-oncogene serine/threonine-protein kinase

CREB – cAMP response element-binding protein

Cys-NAT – cysteine N-acetyl transferase

DAPI – 4',6-diamidino-2-phenylindole

DAT – dopamine transporter

DDIT3 or CHOP – DNA damage-inducible transcript 3

DGAV – Direção-Geral de Alimentação e Veterinária

$DiOC_6(3)$ – 3,3'-dihexyloxacarbocyanine iodide

DMEM – Dulbecco's modified Eagle's medium

DMSO – dimethyl sulfoxide

DnaJ (Hsp40, *HSP40*) – chaperone DnaJ, heat-schock protein 40kDa

DnaK (Hsp70, *HSP70*) – chaperone DnaK, heat-schock protein 70kDa

donorHB – number of donor hydrogen bonds (QikProp)

E2F1 – transcription factor E2F1

ECACC – European collection of cell cultures

ECL – enhanced chemiluminescence

EGCG – epigallocatechin gallate

eIF2a – eukaryotic translation initiation factor 2A

eIF4G – eukaryotic translation initiation factor 4 gamma

EMEM – Eagle's minimal essential medium

ER – endoplasmic reticulum

ERK – extracellular signal–regulated kinase

ESI – electrospray ionization

FBS – fetal bovine serum

FCT – Fundação para a Ciência e Tecnologia

FDA – fluoresceine diacetate

FEDER – European Regional Development Fund

FELASA – Federation of European Laboratory Animal Science Associations

FGF – fibroblast growth factor

FTH1 – ferritin heavy chain

FUS/TLS – RNA binding protein fused in sarcoma/translocated in sarcoma

GA – gallic acid

GADD45A – growth arrest and DNA-damage-inducible protein GADD45 alpha

GAE – gallic acid equivalents

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

GARS – glycine-tRNA ligase

GCLC – glutamate-cysteine ligase

GCLM – glutamate-cysteine ligase

GDNF – glial cell-derived neurotrophic factor

GFAP – glial fibrillary acidic protein

GGT – gamma glutamyl transferase

GLT1 – glutamate transporter 1

GLUT1 – glucose transporter 1

GPX1 – glutathione peroxidase 1

GSH – glutathione

GSK3β – glycogen synthase kinase 3 beta

GSR – glutathione reductase

GSSG – oxidized glutathione

GST – glutathione S-transferase

GSTM1 – glutathione S-transferase Mu 1

GSTP1 – glutathione S-transferase Pi 1

GSTT1 – glutathione S-transferase theta-1

hBDP – hydrolyzed blackberry- driven (poly)phenols

HBMEC – human brain microvascular endotelial cells

HBSS – Hank's balanced salt solution

HD – Huntington disease

HERPUD1 – homocysteine inducible ER protein with ubiquitin like domain 1

HMOX1 – heme oxygenase 1

HPLC – high performance liquid chromatography

HPRT1 – hypoxanthine phosphoribosyltransferase 1

Hsp – heat shock proteins

HSP90AA1 – heat shock protein 90 alpha family class A member 1

HSP90B1 – heat shock protein 90 beta family member 1

HSPA5 – heat shock Ppotein family A (Hsp70) member 5

Htt – huntingtin

IARS – isoleucyl tRNA synthetase

IKK – IκB kinase

IL-1 – interleukin-1

IL-1β – interleukin-1 beta

IL-6 – interleukin-6

IL-8 – interleukin-8

iNOS – inducible nitric oxide synthase

IPA – Ingenuity Pathway Analysis

iPSC – induced pluripotent stem cells

IRS1 – insulin receptor substrate 1

IκBα – nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

JAK – Janus kinase

JAK2 – Janus kinase 2

JNK – c-Jun N-terminal kinases

JUN – c-Jun

Keap1 – Kelch-like ECH-associated protein 1

KEGG – Kyoto Encyclopedia of Genes and Genomes

LBs – Lewy bodies

LC-MS – liquid chromatography–mass spectrometry

LPH – lactase phloridzin hydrolase

LPS – lipopolysaccharide

LUHMES – Lund human mesencephalic

m/z – mass-to-charge ratio

MAP3K5 – mitogen-activated protein kinase kinase kinase 5

MAPK – mitogen-activated protein kinase

MCM – minichromosome maintenance complex

MeDi – Mediterranean diet

MEK – mitogen-activated protein kinase kinase (also known as MAP2K, MAPKK)

MEKK (MAP3K1) – MAP kinase kinase kinase

MEM – minimum essential medium

mhGAP – mental health gap action program

MPP^+ – 1-methyl-4-phenylpyridinium

MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MRP – multidrug resistance-associated protein

MRP1 (ABCC1) – multidrug resistance-associated protein type 1

MRP2 (ABCC2) – multidrug resistance-associated protein type 2

MS – mass spectrometry

MS/MS or MS² – tandem mass spectrometry

mTOR – mammalian target of rapamycin

NAT2 – N-acetyltransferase 2

NDs – neurodegenerative diseases

NEAA – non-essential amino acids

NFAT – nuclear factor of activated T-cells

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NQO1 – NAD(P)H dehydrogenase [quinone] 1

Nrf2 – nuclear factor (erythroid-derived 2)-like 2

NUPR1 – nuclear protein 1

OATP1A2 – organic anion-transporting polypeptide

OATs – organic anion transporters

ORC1 – origin recognition complex subunit 1

ORC6 – origin recognition complex subunit 6

P/S – penicillin / streptomycin

p53 / TP53 – tumor protein p53

PBS – phosphate buffer saline

PD – Parkinson's disease

PDA – photo diode array

PEDF – pigment epithelium-derived factor

PERK – protein kinase R-like endoplasmic reticulum kinase; eukaryotic translation initiation factor 2-alpha kinase 3

P-gp (ABCB1) – P-glycoprotein

PHGDH – phosphoglycerate dehydrogenase

PI – propidium iodide

PI3K – phosphoinositide 3-kinase

Pitx3 – pituitary homeobox 3

PKD – pyruvate dehydrogenase kinase

PLO – poly-L-ornithine

PPAR – peroxisome proliferator-activated receptors

PRKD1 – protein kinase D1

PSA – Van der Waals surface area (QikProp)

PSPH – phosphoserine phosphatase

PSTA1 – phosphate transport system permease protein PstA 1

PTP4A3 – protein tyrosine phosphatase type IVA, Member 3

PVDF – polyvinylidene fluoride

Pyr-sulf – pyrogallol-*O*-sulfate (pyrogallol-1-*O*-sulfate and pyrogallol-2-O-sulfate)

QPlogBB – predicted brain/blood partition coefficient (QikProp)

QPlogKhsa – prediction of binding to human serum albumin (QikProp)

QPlogPo/w – predicted octanol/water partition coefficient (QikProp)

QPPCaco – predicted apparent Caco-2 cell permeability (QikProp)

QPPMDCK – predicted apparent MDCK cell permeability (QikProp)

QREN – Quadro de Referência Estratégica Nacional

RMA – robust multi-array average

RNS – reactive nitrogen species

ROS – reactive oxygen species

RPA – replication proteins A

RPL22 – ribosomal protein L22

RPS6KA2 – ribosomal protein S6 kinase A2

RT-qPCR – reverse transcription quantitative polymerase chain reaction

SARS – seryl-tRNA synthetase

SD – standard deviation

SEM – standard error of the mean

SERPINF1 – serpin F1 or PEDF

SGLT1 – sodium/glucose cotransporter 1

SHMT2 – serine hydroxymethyltransferase

SIRT1 – sirtuin 1

SLC – solute carrier family

SOCS3 – suppressor of cytokine signaling 3

SOD1 – superoxide dismutase 1

SQSTM1 – sequestosome 1

SREBF1 – sterol regulatory element-binding transcription factor 1

SREBP – sterol regulatory element-binding protein

STAT – signal transducer and activator of transcription

Syn (SYP) – synaptophysin

***t*-BHP** – *tert*-butyl hydroperoxide

TDP-43 – TAR DNA-binding protein 43

TEER – transendothelial electric resistance

TGF-β – transforming growth factor beta

TH – tyrosine hydroxilase

TNF-α – tumor necrosis factor alpha

TOR – target of rapamycin

TrkB – tropomyosin receptor kinase B

TXNRD1 – thioredoxin reductase 1

UDP – uridine diphosphate

UDPG – UDP-glucuronosyl transferase

UPR – unfolded protein response

VA-sulf – vanillic acid-*O*-sulfate

VEGF – vascular endothelial growth factor

VMAT2 – vesicular monoamine transporter 2

WARS – tryptophanyl-tRNA synthetase

WHO – World Health Organization

XPOT – exportin-T

$\Delta\Psi$m – mitochondrial transmembrane potential

αSyn – alpha-synuclein

βIII-tub (TUJ1) – βIII-tubulin

Chapter 1

Introduction

Part of this chapter is based on the following manuscript:

Polyphenols Beyond Barriers: A Glimpse into the Brain

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1. NEURODEGENERATIVE DISORDERS

Neurodegenerative disorders (NDs) collectively refer to debilitating, life-threatening conditions that affect brain cells. As chronic and progressive neurological syndromes, they are caused by nervous system dysfunction resulting from neuronal cell failure[1], leading to impaired mental functioning (dementia) or movement complications (ataxia). These diseases can arise from hereditary or sporadic conditions, having a complex pathogenesis that triggers atrophy of central or peripheral structures of the nervous system[2]. Disease-modifying therapies to delay or reverse disease progression are not yet available, there is only a paucity of pharmacotherapy strategies focused on symptomatic relief.

More than 600 disorders have been described to afflict the nervous system such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), multiple sclerosis, brain cancer, degenerative nerve diseases, encephalitis, epilepsy, among others. They are heterogeneous and multifactorial pathologies, which affect different brain structures and have different aetiologies. AD, PD and HD, as well as ALS and others, share the aggregation of misfolded proteins as common pathological processes, being collectively designated as protein conformational disorders[2, 3].

AD is a clinical syndrome characterized by the progressive degeneration of hippocampus and neocortical brain neurons[4], which is responsible for the major disease symptoms – memory loss and cognitive decline. AD pathological hallmarks include the accumulation of extracellular amyloid plaques, majorly composed by amyloid-β peptides (Aβ40 and Aβ42)[5], and intracellular aggregates of hyperphosphorylated forms of tau, a microtubule-binding protein, designated neurofibrillary tangles[6]. AD represents the primary cause of dementia accounting for nearly 70% of known dementia cases and being one of the leading causes of mortality worldwide[7, 8]. The incidence rates vary between 5 and 8 per thousand persons–years, which corresponds to half of new dementia cases each year[9, 10].

PD is the second most common ND. Its clinical symptoms include muscle rigidity, bradykinesia, resting tremor and postural instability, caused by the loss of dopaminergic neurons in the *substantia nigra pars compacta*[11]. The cytoplasmic inclusions designated

Lewy bodies (LBs), predominantly enclosing aggregated α-synuclein (αSyn)[12], are the major pathological hallmark of the disease. αSyn is highly expressed in the brain and its function is thought to be involved in the regulation of dopamine neurotransmission and synaptic function/plasticity[13-18]. Following AD, PD is the second most common ND. The Parkinson's disease Foundation predicts that PD affects 7-10 million people worldwide. The prevalence rates vary from circa 50-300 per 100.000 individuals whereas the incidence rates are about 10-20 new cases per 100.000, annually[19, 20].

HD displays a wide variety of symptoms including chorea, dementia, and emotional disturbance[21, 22]. It is characterized by neuronal demise especially in the striatal region of the basal ganglia. Genetic mutations causing HD are linked to the expression of N-terminal polyglutamine (polyQ)-expanded huntingtin (Htt) beyond a critical length of ~35 glutamine residues. The cleavage of these polyQ tails generates cytotoxic fragments with high propensity to cross-link and form protein aggregates in both neuronal and glial cells[23, 24]. As a rare neurodegenerative condition, the global HD prevalence is 2.71 per 100,000 individuals, being higher in Europe, North America and Australia than in Asia[25].

ALS is a fatal motor neuron disease leading to death usually within 3-5 years after the disease onset, mostly due to respiratory failure. It is characterized by progressive muscle weakness, which frequently starts in the limbs, axial, bulbar, or respiratory muscles and afterwards generalizes relentlessly causing a gradual disability. The disease is caused by progressive loss of cortical, bulbar, and ventral cord motor neurons, with the major genetic risk factors being mutations in the genes encoding the superoxide dismutase SOD1[26], the TAR-DNA-binding protein[27] and the fused in sarcoma or translocated in liposarcoma protein (FUS/TLS)[28, 29]. Consistent with the very limited survival of patients suffering ALS, prevalence is quite low, ranging between 4-5 people out of 100.000 and accounting for 1/300 to 1/400 of all deaths in the US. The median annual incidence of the disease in Europe and the US is estimated at 0.7-2.5 per 100,000 individuals[19, 30].

Besides the determinant protein aggregation role in major NDs, several other factors are common and transversal to them. As such, in the past years, a new paradigm has been emerging to devise interventions which target common mechanisms of ageing to delay the onset of more than one age-related disease at the same time[31]. Disease

hallmarks like increased oxidative stress, protein carbonylation, lipid peroxidation, DNA damage, mitochondrial dysfunction, vascular dysfunction, endoplasmic reticulum (ER) stress, unfolded protein response (UPR) dysregulation and imbalanced proteostasis, and also neuroinflammation are common and will require mutitargeted interventions to tackle disease progression. Importantly, neuroinflammation appears as one of the key processes involved in major NDs: neuroinflammation has been regarded as a double-edged sword, being not only essential for the recovery from a number of conditions, but it may also play detrimental roles in neurodegenerative processes, contributing to disease progression. Altogether, this information reinforces the concomitant burden that NDs present in the developed world and the trend to increase over time. The need for novel therapies assisting to retard and prevent the development of these diseases is imperative and must be the focus of research in this area, rather than finding new alternatives to treat symptoms in later stages of disease progression.

Though ageing is considered a primary process in neurodegeneration, it differently influences the onset of the various NDs. Advancing age is the leading risk factor for AD, and disease onset usually occurs at the age of 65[7]. Reports indicate that the risk of developing this disease almost doubles every five years after this age[9, 10]. Likewise, PD majorly afflicts individuals over 60 with higher median prevalence rates over 65s than in the overall population of US and European countries[19]. As a consequence, median PD incidence ratios are also much higher in individuals over the 65s (160 per 100,000 person-years) than in the overall population (14 per 100,000 person-years)[19]. Indeed, it is estimated that the development of treatments reducing severe cognitive impairment in older people by just 1% per year would cancel out the projected increases in the long-term care costs due to our ageing population (Alzheimer's Research Trust).

1.1. Healthy ageing and transition to disease

Ageing is a complex, irreversible, progressive and natural process, which is characterized by morphological, psychological, functional and biochemical changes, affecting the welfare and health of the individual. The maximization of the functional capacity and health of the elderly is conditioned by factors such as nutrition, physical

and social activity, education, and genetic background, which ultimately define healthy ageing. Some of these factors are not modifiable, such as genetic ones, while others (nutrition, physical and social activity, *etc*.) are subject to change (*i.e.* environmental, psychological, social and lifestyle). In this sense, nutrition and other modifiable factors have a huge impact on health and wellbeing. The nutritional status of elderly people has been increasingly considered a key aspect for a healthy ageing and, therefore, nutrition emerges as a critical modifiable risk factor to be exploited in policy strategies to prevent or delay the onset of NDs and dementia[32]. There are several evidences that a continuous and prolonged intake of fruit and vegetables, rich sources of compounds named (poly)phenols, may help in the prevention of several degenerative pathologies such as diabetes, cardiovascular diseases, NDs and cancer, and to prevent symptoms associated to ageing and menopause[33, 34].

If we consider the normal process of evolution of a healthy state to a disease state, we may resume that the evidences indicate an active role of dietary (poly)phenols to homeostasis maintenance, delaying or even reversing the transition from a healthy to a pathological state (**Fig. 1**). Then, nutrition, and in particular bioactive (poly)phenols identified in the diet, are strong contributors to the maintenance of a healthy condition. Moreover, (poly)phenols can also constitute lead compounds as basis for developing new drugs and therefore contribute to a pharmacology intervention (**Fig. 1**). Thus, pharmacological and nutritional approaches can be considered for the study of (poly)phenols in a neuroprotection perspective.

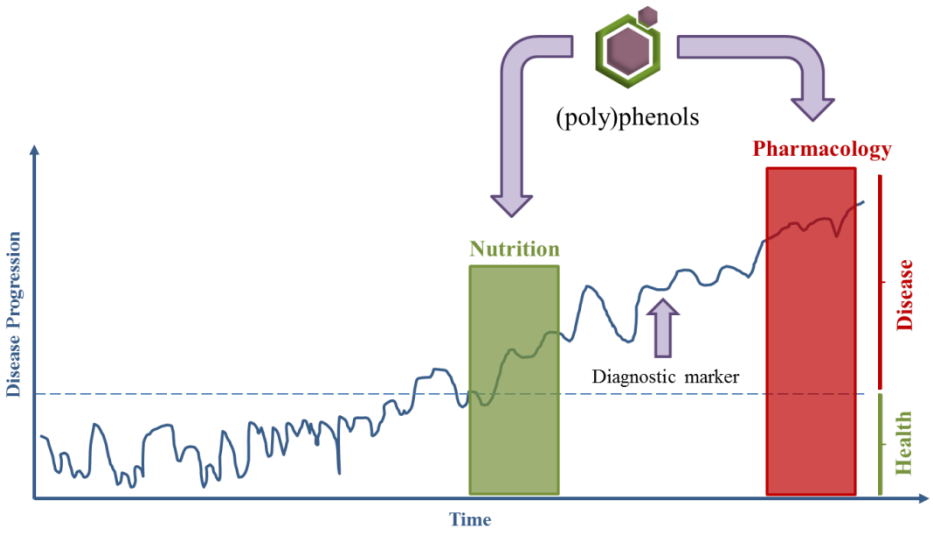

Fig. 1 Disease progression and transition from healthy to disease state. (Poly)phenols can potentially act in the prevention of disease, through nutrition, or to restore the healthy state in the earlier stages of a disease even before the administration of a drug. Drugs are only prescribed after a diagnostic marker has been identified and generally treat only the symptoms of degenerative diseases and are not a cure in most cases. (Poly)phenols can also be the basis for developing new therapeutic compounds and therefore contribute for a pharmacology intervention.

2. (POLY)PHENOLS AND HEALTH

Phenolic compounds, commonly referred as polyphenols, constitute one of the most extensive and ubiquitous group of secondary metabolites in the plant kingdom. These compounds are characterized structurally by the presence of, at least, one hydroxyl functional group (-HO) linked to an aromatic ring[35]. Some compounds that do not present structural characteristics of polyphenols are commonly integrated in the group of polyphenols as "honorary", such as phenolic acids or stilbenes. For this reason, recently the term "polyphenols" has been rewritten as "(poly)phenols"[36-38].

(Poly)phenols represent an extremely differentiated group not only in terms of chemical structure but also in terms of their biological activity. They occur conjugated with sugars, carboxylic and organic acids, amines, lipids and other phenols. Different groups are classified in terms of the number of phenol rings that they encompass and according to the structural elements binding these rings[39, 40]. The main classes are represented in **Fig. 2** and include phenolic acids, stilbenes, flavonoids, coumarins and lignans.

Hydroxybenzoic and hydroxycinnamic acids represent the two different groups of phenolic acids. The first ones can be found in plants, both free and esterified. Examples are gallic acid, present in fruits, herbs, tea and wine, or more complex compounds, the hydrolysable tannins such as ellagic acid, gallotannins and ellagitannins[41]. Hydroxycinnamic acids are generally represented by *p*-coumaric, caffeic, ferulic and sinapic acids, usually found glycosylated or conjugated with quinic, shikimic and tartaric acids[39, 41]. Chlorogenic acid, an ester of caffeic and quinic acids, can be found in several fruits and vegetables and is highly abundant in coffee. Ferulic acid is present in large amounts in cereal grains[41, 42]. Stilbenes frequently present in roots, barks, rhizomes and leaves, are not routinely consumed. By opposition we have a highly valued stilbene, resveratrol, present in grapes and in red wine[43].

The largest group of phenolic compounds in plants are flavonoids, with more than 10,000 different structures being identified[44]. The main classes of flavonoids are flavonols, flavones, isoflavones, flavanones, anthocyanins and flavanols. Monomers of flavanols (catechins), such as (+)-catechin and (-)-epicatechin, are relatively abundant in fruits, wine, chocolate and green tea[41, 45]. Proanthocyanidins, also known as condensed tannins, are constituted by dimers, oligomers and polymers of catechins. Proanthocyanidins can be found in fruits such as apples and grapes, in wine, cider, tea and beer, and also in cocoa[46]. Anthocyanins are glycosylated pigments, responsible for the colours of some flowers and fruits.

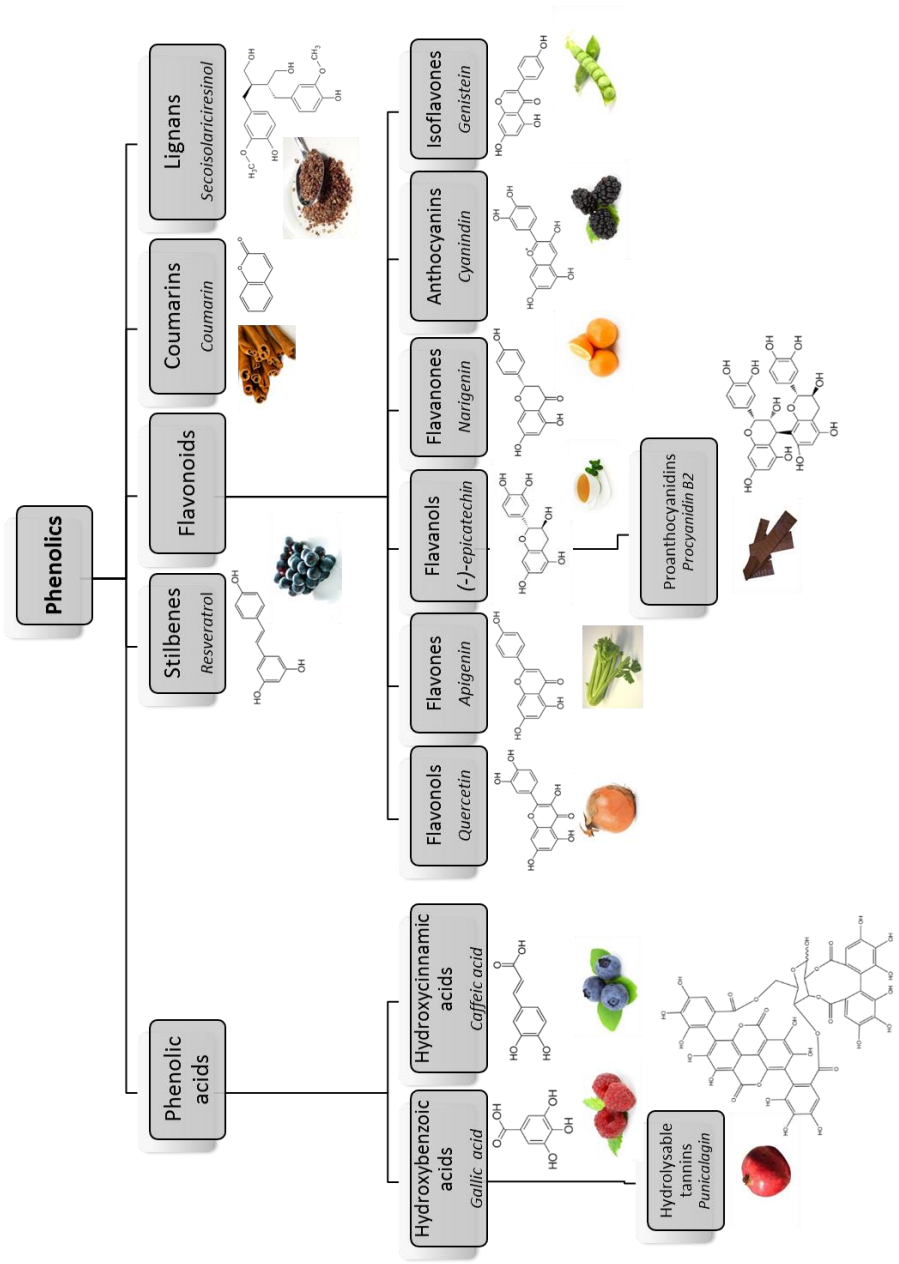

Fig. 2 Main (poly)phenol classes with structure, name of representative compounds (in italic) and examples of food sources are represented.

2.1. Cognitive health and dietary (poly)phenols: epidemiological and population based studies

The beneficial effects resulting from (poly)phenol intake have been extensively studied, as in the studies concerning the Mediterranean Diet (MeDi). MeDi is characterized by a high consumption of fruits, vegetables, and grains, as well as sea-fish on regular bases. It also includes a modest consume of wine and olive oil as the principal source of fat, both highly enriched in (poly)phenols. Additionally, the intake of meat, dairy products, sweets and convenience food is rather low in the MeDi[47, 48].

A survey of studies reporting the effects of MeDi, carried out by WHO, revealed that it is a promising strategy to prevent diseases and to enhance quality of life (World Health Organization, 2009). Furthermore, epidemiologic studies over the last decades have supported the positive correlation between Mediterranean eating patterns and a large number of health benefits[49, 50], including (i) decreased risk of developing NDs; delayed AD and PD onset; (iii) lowered mortality in AD patients; and (iv) improved cognitive function[32, 51-53].

(Poly)phenols are abundant in MeDi and are believed to contribute to the beneficial effects of this diet when adopted in a regular basis, as revealed by studies showing that (poly)phenol-rich diets improve cognition, memory, learning, and vascular function in elderly people[54, 55]. The regular consumption of flavonoid rich-foods, representing the most common group of (poly)phenolic compounds in the human diet, has been associated to enhanced cognitive abilities and reduced risk of cognitive decline in aged individuals[56, 57]. Indeed, a large-scale population study indicated that flavonoids intake decreased dementia as well as premature death due to dementia[57]. Remarkably, it was found a correlation between high (poly)phenol concentrations in urine samples of older adults and lower risk of cognitive decline in global cognitive function in a prospective population-based study over a 3-year period[58].

Among the different food sources, berries are considered to be a rich source of (poly)phenols with brain health benefits. In fact, wild blueberry diet supplementation was proved to improve cognitive function in older adults[59]. Increased consumption of berries was shown to be associated to a slower progression of cognitive decline in a large prospective cohort of older women[60]. Also, it was demonstrated that high anthocyanin

consumption is associated with reduced risk of developing PD[61]. A recent study have shown that a chronic blueberry supplementation improved brain perfusion, task-related activation, and cognitive function in healthy older adults, highlighting that a diet supplementation with an anthocyanin-rich concentrate can improve brain activation in brain areas associated with cognitive function[62]. Moreover, in a double-blind, placebo controlled trial, the addition of easily achievable quantities of blueberry to the diets of older adults improved some aspects of cognition[63].

These studies support the hypothesis that (poly)phenol-rich foods or supplements have a positive impact towards NDs. How it is processed and by which mechanisms diet alterations may exert protective effects is still a field of intensive research that, in the future, may change our perspective of an effective treatment for NDs.

2.2. Nutritional relevance and bioavailability of (poly)phenols

Although (poly)phenols are not essential for humans, they have a positive impact on human nutrition. (Poly)phenols are widely spread in food, and the total (poly)phenols dietary intake could be as high as 100-150 mg per day, which is much higher than that of all other classes of phytochemicals[41]. Just for perspective, this is one order of magnitude higher than the intake of vitamin C and two orders of magnitude higher that the intake of vitamin E and carotenoids[64, 65]. (Poly)phenols' main dietary sources are fruits and plant-derived beverages such as fruit juices, tea, coffee, and red wine. Vegetables, cereals, chocolate, and dry legumes also contribute to the total (poly)phenol intake[41]. The amount of research that has emerged in the past years in order to better understand (poly)phenols health benefits discloses a glimpse of the huge potential they may present[66-68]. However, for a deeper understanding of the effect of (poly)phenols in human health, their absorption, distribution, metabolism and excretion in the human digestive tract needs to be studied.

The most common (poly)phenols present in the human diet are not necessarily the most active inside the body, either due to a low inherent activity or due to their poor absorption, extensively metabolism and rapid excretion[41]. Throughout digestion, (poly)phenols suffer several chemical modifications and metabolism, and the bioavailable metabolites found in blood and tissues may diverge from the native

compounds in terms of biological role. Many different models are used to obtain a deeper knowledge about these mechanisms, ranging from *in vitro* enzymatic activities, cellular models, animal models or even the man himself. Although many differences can be seen between human and other animals' digestive process, animal studies have been essential for the current understanding gathered so far on (poly)phenols bioavailability and effects.

One of the major difficulties on studying the bioavailability of (poly)phenols relies on their structural differences, resulting in different metabolic fates among compounds. Although some reactions could be common, several differences in the (poly)phenols metabolism can occur among classes or even within the same class.

After ingestion, the availability of (poly)phenols, often associated with fibre or complex carbohydrates in the food matrix, can be modified in the oral cavity by amylase digestion and, perhaps, by particle size reduction[69]. Afterwards, the effective release of the phenolic compounds in the stomach maximizes the potential for absorption in the small intestine[69]. Absorption occurs mainly at the duodenum and at the proximal half of jejunum, where enterocytes are the predominant cells, being responsible for the absorption (**Fig. 3**). Being lipophilic compounds, most flavonoid aglycones and phenolic acids permeate intestinal cells by passive diffusion[33, 70, 71]. (Poly)phenols in the form of esters, glycosides or polymers, usually present in plants, cannot be directly absorbed and they probably resist to acid hydrolysis in the stomach, being able to reach the duodenum[41].

The absorption of glycosylated compounds is usually preceded by the hydrolysis of the glycoside and release of the aglycone (**Fig. 3**), by the enzyme lactase phloridzin hydrolase (LPH) in the brush-border of the small intestine epithelial cells. After hydrolysis, the free aglycone can enter the epithelial cells by passive diffusion[72]. Otherwise, glycosylated compounds are supposed to enter epithelial cells via the sodium-dependent glucose transporter, SGLT1, and be hydrolyzed by the cytosolic β-glucosidase (CBG), depending on the glycoside[73]. The highly stable oligomeric and polymeric flavanols, during digestion, are thought to have a very low absorption[74].

Compounds that are not absorbed in the proximal gastrointestinal tract can reach the large intestine where absorption can also happen (**Fig. 3**). There, colonic microbiota can

degrade complex (poly)phenols into low molecular weight phenolics, aromatic acids[75], and also into oxaloacetate and CO_2[76].

As usually happens with xenobiotics, (poly)phenols can undergo enzymatic modifications at the liver known as 'biotransformations', phase I and phase II reactions (**Fig. 3**)[77]. During phase I metabolism, oxidation reactions (mainly via cytochrome P450), reduction, and hydrolysis reactions occur, converting (poly)phenols into more polar (water soluble) metabolites by unmasking or inserting a polar functional group (-OH, -SH, -NH_2). Further conjugation reactions that increase water solubility of drug with a polar moiety take place during phase II metabolism, mainly glucuronidation, acetylation, and sulfation reactions. The resulting compounds are frequently less lipophilic so the body can more easily excrete them.

Phase III transporters are responsible for the final step in the elimination and/or detoxification of xenobiotics, resulting in the removal of the undesired compounds in faeces, urine and bile[78] (**Fig. 3**). These transporters are mainly constituted by the ABC protein family, including P-glycoprotein (P-gp), the multidrug resistance-associated proteins (MRP2, BCRP), and also by the solute carrier family (SLC) transporters[79, 80].

Importantly, most of the conjugated-(poly)phenols are excreted in urine. However, the mechanisms by which these conjugated-(poly)phenols can return into the intestinal lumen or to bile, by the action of intestinal cells or liver parenchymal cells, respectively, should also be considered (**Fig. 3**). Several (poly)phenols were shown to be considerably better absorbed in the presence of additional (poly)phenols, interfering with efflux transporters (P-gp, MRPs, BCRP), which normally reduce the intracellular concentration of such "xenobiotics" and, in that case, lead to their excretion to the apical side[81, 82]. On the other hand, transporters to the basolateral side are poorly understood. Compounds excreted into bile are eventually emptied in the duodenum. Usually, they are not absorbed in the small intestine, and reach the colon, where they can either be excreted into the faeces or be degraded by the colon microbiota, and be ultimately reabsorbed[83].

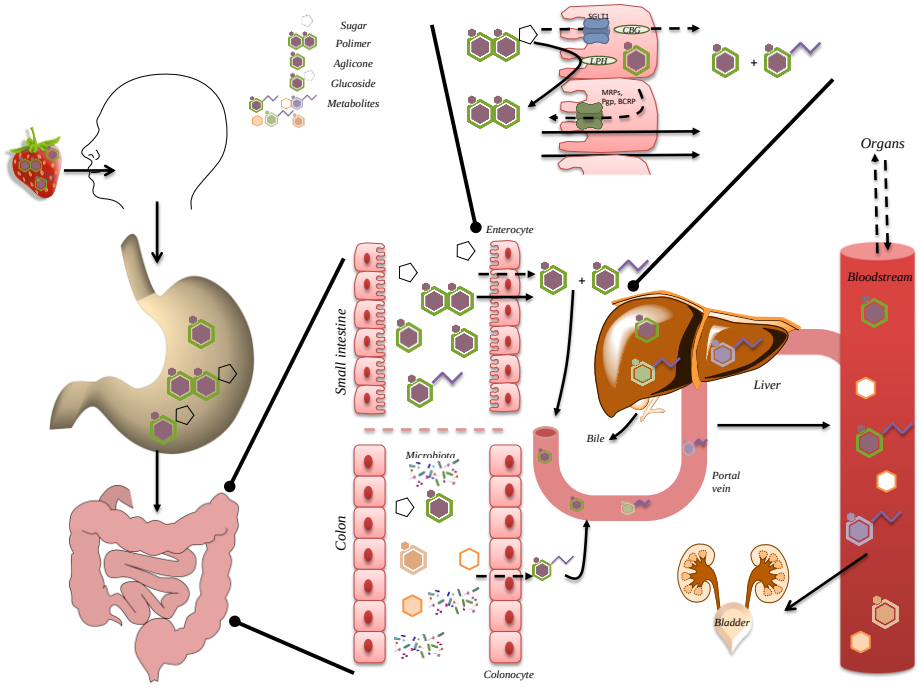

Fig. 3 Schematic representation of absorption, biotransformation and excretion of (poly)phenols in the human body. Along the digestive process, (poly)phenol-rich food suffers transformations, starting in the mouth, stomach and throughout the entire gastrointestinal tract. The gastrointestinal tract is covered by the mucosa, covering intestinal epithelium which functions as a physical barrier, determining bioavailability of xenobiotics like (poly)phenols. This function is mediated by physical walls, metabolism and passive (solid arrows)/active (dashed arrows) transport mechanisms. Absorption occurs mainly at the duodenum and the proximal half of jejunum, in enterocytes. Enterocytes apical cell membranes contain microvilli, which increase the surface area of absorption. Passive intestinal permeability occurs mainly for aglycones and simple phenolic acids. Absorption of glycosylated compounds is usually preceded by release of aglycone through hydrolysis by lactase phloridzin hydrolase (LPH). Free aglycone can then enter the epithelial cells by passive diffusion. Alternatively, glycosylated compounds enter epithelial cells by the active sodium-dependent glucose transporter SGLT1 and are hydrolyzed by the cytosolic β-glucosidase (CBG). Once inside enterocytes, (poly)phenols can be extruded into the lumen by efflux transporters (P-gp, MRPs, BCRP). Compounds not absorbed reach the colon where they can be extensively metabolized by microbiota. Several transformations in (poly)phenols structure can occur. Most of the colonic metabolites are excreted in feces, although absorption can still take place. Then, (poly)phenols can undergo phase I and phase II reactions. Phase I reactions include oxidative and reductive reactions. Glucuronidation, sulfation and methylation are the most frequent phase II reactions. The conjugates, being more water soluble, are rapidly excreted through bile or urine [77]. Metabolites can then be transported into the bile (enterohepatic recirculation) and secreted back to the duodenum. Degradation of metabolites in the intestine generates catabolites available for reabsorption.

Bioavailability of (poly)phenols is thus a multi-stage process comprising de-conjugation and possible catabolism, absorption, conjugation and excretion (**Fig. 3**). Moreover, the possible sequestration of some (poly)phenol metabolites inside tissues has been recurrently undetermined and may contribute to underestimation of the their

bioavailability. The accessibility of (poly)phenol metabolites to the central nervous system is an example where the presence of an additional barrier, the blood-brain barrier (BBB), reduces even more (poly)phenols bioavailability, in addition to the described metabolism. Therefore, controlled target delivery to central nervous system (CNS) is desirable to optimize (poly)phenols in terms of efficiency, specificity and safety[84, 85].

3. NEUROPROTECTIVE POTENTIAL OF (POLY)PHENOLS

In addition to the epidemiological evidences already mentioned, neuroprotective evidences for (poly)phenols also result from *in vitro* (cell free assays), cellular, animal and clinical studies[54, 86]. Another aspect important to consider is that (poly)phenols can be studied either in a pharmacological or a nutritional perspective. Besides the form of delivery/route of administration, we can consider studies focusing on pure compounds, namely studies with (poly)phenol aglycones (**Table 3**), with relevance in a pharmacological context, or with (poly)phenol metabolites (**Tables 2, 4, 5**), or even with the use of mixtures or whole extracts (**Table 1**), highlighting (poly)phenols potential in a more nutritional perspective.

Neurodegeneration is a multifactorial process and (poly)phenols present pleiotropic effects (antioxidant, anti-inflammatory, immunomodulatory properties)[87] due to their ability to modulate the activity of multiple targets involved in pathogenesis, thereby potentially halting the progression of these diseases. For many years, (poly)phenols were thought to protect cell constituents against oxidative damage through direct scavenging of free radicals. Such idea has become very popular leading to the appearance of several studies exploring extensively this property of (poly)phenols for NDs, since oxidative stress constitutes an important hallmark of these diseases. However, this concept now appears to be an oversimplified view of their mode of action. There is an emerging acceptance that (poly)phenols, as well as their metabolites, exert modulatory actions in proteins/enzymes through direct interaction with receptors or

enzymes involved in signal transduction, such as protein kinase and lipid kinase signalling pathways[88]. Moreover, several neurochemical mechanisms underlying the protective action of (poly)phenols have been described: iron chelating properties[89]; modulation of signalling pathways related with neuronal survival and differentiation[88, 90]; inhibition of neuropathological processes[91, 92]; and regulation of mitochondrial function[93-95]. Other mechanisms by which flavonoids can be neuroprotective include their positive role on peripheral and cerebrovascular blood flow, ultimately affecting synaptic plasticity processes and cognitive function[96]. Accumulating evidence highlights the molecular and cellular pathways mainly modulated by parent (poly)phenols, which, though not always nutritionally relevant, can give us clues about the putative mode of action of their (poly)phenol metabolites in a nutritional context. The study of the neuroprotective potential of (poly)phenol metabolites resulting from extensive human metabolism (**Fig. 3**), however, is still in its infancy and should be further explored.

Importantly to notice, the study of pure compounds is limited to their molecular mechanisms in neurodegeneration cell models and their specific biological effects in animal models and such studies miss the synergies of different (poly)phenols. The overall diet complexity, the potential synergy that may occur in compounds derived from the metabolism cannot be fully depicted in studies using pure compounds[97]. Studies with (poly)phenol-enriched fractions or with the bulk of digested/metabolized compounds may be useful to approach this interactions and depict the molecular effects of the overall metabolites in cells. Since (poly)phenols and (poly)phenol metabolites are emerging as key compounds for the development of novel therapeutic agents for NDs, the identification of the molecular targets taking advantage from *in vitro* (cell free) studies, cell models and *in vivo* studies has become an emerging area of research (**Tables 1-5**).

3.1. *In vitro* (cell free) studies

The consensus on the important role of free radical-mediated reactions in the pathophysiology of most NDs lead to the extensive study of (poly)phenols chemical scavenging activity[98-101].

Table 1. Neuroprotective evidences (*in vitro*, cellular models and animal studies) for the most representative dietary (poly)phenolic extracts.

	In vitro (cell free)	Cellular model	Animal model
Green tea	∗ Scavenged ROS/NOS [100] ∗ Inhibited AChE [102]	∗ Scavenging intracellular RNOS and induced endogenous antioxidant defences preventing DNA damage [100] ∗ Protects primary rat cortical neurons against Aβ-induced cytotoxicity [103]	Ageing and neurodegeneration models: ∗ ↓ Protein/lipid oxidation [104] ∗ ↑ Spatial learning [104] ∗ Modulation of glutathione levels and antioxidant enzyme activities [105] ∗ ↑ CREB activation [105] ∗ ↑ BDNF and Bcl-2 levels [105] ∗ ↑ Cognitive and behavioural capacities [106] ∗ Protects against deltamethrin-induced neurotoxicity in rat [107] AD rodent models: ∗ ↓ Aluminium chloride toxicity [108] PD non-human primates model: ∗ Alleviate motor impairments, dopaminergic neuronal injury, and aSyn aggregation [109]
Grape seed extract and derivatives	∗ Inhibited Aβ aggregation and cytotoxicity [110]	∗ ↑ IL-6 and respective mRNAs in primary culture of astrocytes, which functions as a neuroprotective paracrine, protected neuronal cells from death by oxidative stress [111] ∗ Protects neuronal cells against low extracellular Mg^{2+} concentration and oxygen glucose deprivation-induced neurotoxicity, in cultured rat hippocampal neurons mediated by inhibition of glutamate-induced calcium signaling and NO formation [112]	AD rodent model: ∗ ↓ cognitive deterioration [92] ∗ ↑ cognitive function [113] ∗ ↓ oligomerization of Aβ peptides and amyloid plaques [92] ∗ ↓ microglial activation [114] ∗ ↓ extracellular-signal-regulated kinases (ERK) 1 and 2 in the brain, suppressing tau neuropathy [115] ∗ Interferes with the assembly of Aβ peptides into neurotoxic aggregates [116] ∗ ↑ Spatial memory performance, ↓ cognitive deterioration and Aβ neuropathy [117] HD rodent model: ∗ Neuroprotective following oral administration [118]
Berries and anthocyanin-rich extracts	∗ Inhibit the formation of Aβ peptide fibrils [119-122]	∗ ↓ Toxicity of Aβ aggregates toward Neuro2a cells [122] ∗ ↑ Aβ aggregates clearance, ↓ fibrillation and supressed microglia activation, in murine cell cultures [123, 124] ∗ Protection of neuronal cultures from oxidative stress [125] ∗ ↓ Neuroinflammation [126] ∗ ↓ Neuronal death in cells expressing Aβ by improving cellular metabolism [127]	Ageing and neurodegeneration rodent models: ∗ Reverse age-related deficits in spatial working memory (↑CREB activity, ↑BDNF, hippocampal Akt phosphorylation, activation of TOR and ↑ expression of Arc/Arg3.1 [128] ∗ Delayed age-related motor and cognitive behavioural deficits [129-132] AD rodent model: ∗ ↓ cognitive degeneration [122] ∗ Reverses Aβ-induced effects on protein expression: mitochondrial apoptotic pathway (Bax, cytochrome C, caspase-9 and 3) and AD markers (Aβ, APP, P-tau and BACE-1) [127] ∗ Pomegranate juice oral intake improved spatial learning, and reduced Aβ plaques [133]

Substantial presynaptic cholinergic deficit is a feature registered in most NDs[134]. Malfunction of the cholinergic system may be tackled pharmacologically through

inhibition of acetylcholinesterase (AChE)[135], which catalyses the hydrolysis of the neurotransmitter acetylcholine (ACh) to choline. Therefore, AChE inhibition has been reported to ameliorate the symptoms of some NDs and has been used as a rationale to develop drugs to treat AD[136]. Interestingly, green tea and white tea digested metabolites were described to inhibit AChE[102].

Another common pathological hallmark of many NDs is the generation of aberrant misfolded proteins with formation of intra- or extra-cellular high-ordered insoluble fibrils deposits[137, 138]. Potent activities towards the several steps of fibrils formation are modulated either by extracts or by isolated compounds. For instance, inhibition of Aβ-peptide aggregation and fibril formation was described for blueberry anthocyanins enriched extract[122]; epigallocatechin gallate (EGCG)[139, 140]; myricetin[141] and resveratrol derivatives[119-121, 142, 143].Moreover, the ability to destabilize preformed fibrils *in vitro* was also described for curcumin[144], catechins and procyanidins[145]. Similarly, the protein αSyn is an amyloidogenic polypeptide that forms cytotoxic oligomers and quercetin was described to reduce αSyn fibrillization[146]. It was also shown that EGCG redirect the aggregation of αSyn monomers and remodel αSyn amyloid fibrils into disordered oligomers[139, 147]. In addition to (poly)phenol parent compounds, a study have shown that brain-accumulating phenolic acids, derived from colonic metabolism (namely 3-hydroxybenzoic acid and 3-(3´-hydroxyphenyl)propionic acid), also potently interfere with aggregation of Aβ peptides, *in vitro*[148]. Moreover, the brain-targeted bioactive dietary quercetin-3-O-glucuronide was shown to be capable of interfering with the initial protein-protein interaction of Aβ(1-40) and Aβ(1-42) that is necessary for the formation of neurotoxic oligomeric Aβ species[149]. Urolithins, the ellagitanins physiologically-relevant gut microbiota-derived metabolites, have also shown to prevent Aβ fibrillation *in vitro*[150].

In vitro cell free studies are particularly relevant to determine the effect of (poly)phenols and (poly)phenol metabolites in specific pathological processes, without the interference of other cellular pathways. However, *in vitro* findings must be interpreted with caution, as some are not translatable into cell and animal models. For instance, (poly)phenols present a very promising *in vitro* antioxidant capacity, leading to the misconception that their benefits were mainly due to direct antioxidant scavenging. Studies with cellular and

animal models were valuable to demystify this concept, and now we know that the mode of action of (poly)phenols go far beyond than their antioxidant potential[151].

Table 2. Neuroprotective evidences (*in vitro* – cell free studies) for some bioavailable (polyphenol metabolites.

(Poly)phenol metabolite(s)	*In vitro* test	Neuroprotective evidence	Reference
urolithin A urolithin B pyrogallol dihydrocaffeic acid dihydroferulic acid feruloylglycine 3-hydroxyphenylacetic acid 3,4-dihydroxyphenylacetic acid 3-methoxy-4-hydroxyphenylacetic acid	Albumin (BSA) + glucose	Ellagitannin-derived catabolites (urolithins and pyrogallol) are the most effective antiglycative agents	Verzelloni *et al.* 2011[152]
quercetin-3-O-glucuronide malvidin-3-O-glucoside	Photo-induced cross-linking of unmodified protein assay w/ Aβ(1-42) and Aβ(1-40)	The brain-targeted bioactive dietary quercetin-3-O-glucuronide interfere with the initial protein-protein interaction of Aβ(1-40) and Aβ(1-42) that is necessary for the formation of neurotoxic oligomeric Aβ species	Ho *et al.* 2012[149]
punicagalin punicalin ellagic acid gallic acid urolithin A urolithin B methyl-urolithin A methyl-urolithin B	Thioflavin-T assay w/ Aβ(1-42)	Inhibition on Aβ(1−42) fibrillation	Yuan *et al.* 2015[150]
3-hydroxybenzoic acid 3-(3´-hydroxyphenyl)propionic acid	Photo induced cross-linking of unmodified protein assay Circular dichroism Thioflavin-T assay Electron microscopy w/ Aβ(1-42)	3-hydroxybenzoic acid and 3-(3´-hydroxyphenyl)propionic acid potently interfere with the assembly of β-amyloid peptides into neurotoxic β-amyloid aggregates that play key roles in AD pathogenesis	Wang *et al.* 2015[148]

3.2. Cellular models

Cellular models are very important to unravel the molecular mechanisms underlying the (poly)phenol protective effects observed in animals. They allow to study the cross-talk between pathways affected by (poly)phenols and (poly)phenol metabolites, giving an integrated view of the metabolic pathways affected by these compounds. In contrast to cell free studies, focused on a particular aspect or protein/enzyme function, in cellular

models the whole cellular metabolism is evaluated. Studies analysing the potential of a mixture of compounds, as food extracts, (**Table 1**) can present advantages, such as the existence of potential synergisms between (poly)phenols components (as it occurs in our diet, where (poly)phenols are embed in a food matrix), but over caution should be taken when interpreting the nutritional and biological relevance of such studies: conclusions taken from the usage of conventional food extracts directly on cells may not be translatable to real potential beneficial effects. The usage of extracts resulting from *in vitro* digestion methods can present an elegant approach to obtain (poly)phenol compounds' mixtures more close to the *in vivo* situation. Such mixtures of (poly)phenol components resulting from in vitro digestion are more appropriate for being used in cell studies than conventional extracts when the aim of the studies is to evaluate the potential of complex mixtures of compounds, as equivalent to our diet.

A plethora of mammalian cell models have been used to study the protective action of (poly)phenols towards NDs. Some of these models cover common disease hallmarks such as oxidative stress, DNA damage, lipid peroxidation, and mitochondrial dysfunction. On the other hand, more complex models address particular pathological processes associated to each disease as well as toxicity of specific disease proteins.

Given the close association between inflammation and NDs, a special attention has been given to the use of reliable cellular models to access the anti-inflammatory potential of (poly)phenols for neurodegeneration. In fact, inflammation is a defence mechanism against insults, designed to remove noxious agents and to inhibit their detrimental effects. In neurodegenerative diseases, neuroinflammation may be triggered by external signals arising from systemic inflammation, by the accumulation of proteins with abnormal conformations or by signals emanating from injured neurons. Altered expression of different inflammatory factors can either promote or counteract neurodegenerative processes. By opposition to normal conditions, where microglia activation promotes the resolution of inflammatory damages caused by external agents, in a neurodegeneration scenario, an over-activation of microglia is observed, leading to a chronic state of neuroinflammation, which is deleterious and toxic for the cells. The sustained release of pro-inflammatory mediators' production became harmful to cells and, for that reason, an existence of a shift to an anti-inflammatory state, guided by

cytokines and neurotrophic factors, it is crucial to efficiently repair the damage and restore the mediator's balance, downregulating the inflammation. In the past few years, (poly)phenols potential in modulating this chronic neuroinflammation has also been explored.

Different cell lines have been used to explore therapeutic molecules attenuating inflammation induced by lipopolissacharide (LPS) or tumor necrosis factor α (TNF-α). Since microglia represents the CNS resident innate immune cells, microglial cell lines such as N9 and BV2 have been the preferred ones. Increasing evidences suggest that flavonoids inhibit the production of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1 in BV2 microglial cells, suggesting its close involvement in pathways such as NF-κB or MAPK[126]. In addition, there is strong evidence that blueberry (poly)phenols inhibit the production of NO, IL-1β and TNF-α in activated BV2 cells[123]. Studies evaluating (poly)phenols metabolites effects against neuroinflammation are starting to emerge as well. The potential to reduce LPS-induced TNF-α secretion in THP-1 monocytes was evaluated, demonstrating that some metabolites of flavonoids, individually and in combination, appear to present more anti-inflammatory effects then their precursors[153]. A more recent study from Esteban-Fernández et al. pointed out the effects of wine-derived human metabolites, at physiological concentrations, in protecting neuroblastoma cells from nitrosative stress injury[154]. However, the modulatory effects of (poly)phenol metabolites in microglial cells, the brain resident macrophage cells, which act as the first and main form of active immune defence in the CNS and which are mainly responsible for neuroinflammation modulation is still very scarce.

Despite of the growing evidence which suggests that metabolism of (poly)phenols may actually increase their biological activity, the extensive research with single parent (poly)phenols also revealed targeted potential neuroprotective actions which can provide valuable clues for further studies with relevant circulating metabolites. As such, **Table 3** summarizes the evidences regarding parent (poly)phenols potential against NDs.

Table 3. Neuroprotective evidences (*in vitro*, cellular models and animals) for the most representative aglycones of parent (poly)phenols.

	In vitro (cell free)	Cellular model	Animal model
Cathecins (mainly EGCG)	* Prevent Aβ fibril formation [139] * Redirect the aggregation of aSyn monomers and remodel aSyn amyloid fibrils into disordered oligomers [139, 147]	* ↑ neuronal viability, modulation of signal transduction pathways and mitochondria functions [16, 155] * ↓ neurotoxins and Aβ toxicity in cell lines and primary cultures [106, 156-160] * ↓ caspase activation mediated by Aβ in hippocampal neurons [157]	Ageing and neurodegeneration rodent models: * Prevention of spatial learning and memory decline [29] * ↑ life span [161]; * Prevention brain inflammation [162] AD rodent model: * ↓ amyloidosis [91]; * Rescue memory impairment (↓NF-κB pathway and ↓oxidative stress [163] * Restored mitochondria function in the brain hippocampus, cortex and striatum [164] MS rodent model: * Neuroprotective effects by modulating neuroinflammation and attenuating neural damage [165] Cerebral ischemia rodent model: * Ameliorated redox imbalance and limited inflammation [165] * EGCG improved age–related cognitive decline and protected against ischemia/reperfusion [166]
Resveratrol	* Inhibited the formation of Aβ peptide fibrils [119-122]	* ↓ Aβ toxicity [167] * Modulation of NF-κB and SIRT1 pathways in cell models [167-169]	AD rodent model: * ↓ formation of amyloid plaques, without affecting APP levels [170] * Protection from Aβ neurotoxicity by inhibiting iNOS [171] * ↓ hippocampal neurodegeneration [168] PD rodent model: * ↓ neural inflammation (↓ mRNA levels of COX-2 and TNF-α in the *substantia nigra*) [172] * ↓oxidative stress, lipid peroxidation, and protein carbonyl [173] HD rodent model: * SIRT1 activation [174] MS rodent model: * ↓ neural damage (↑ SIRT1) [175] * Prevention neural loss without immunosuppression [176] Cerebral ischemia rodent model: * Improve brain energy metabolism [177] * Modulation of the release of neurotransmitters and neuromodulators [178] Non-human primate study: * supplementation increased spatial memory performance [179]
Curcumin	* Destabilized preformed fibrils [144]	* Intracellular antioxidant activities and anti-amyloid activities [7, 180] and MPTP protective activity [181] * anti-inflammatory (↓COX-2) in both rat primary microglial and murine BV2 microglial cells [182] * ↓ iNOS and inhibition of NF-κB and AP-1 activation [183]	AD rodent model: * ↓ Aβ plaques, oxidized proteins and Interleukin-1 beta (IL-1β) [144, 184] HD rodent model: * Counteract huntingtin aggregates formation and partial improvement of transcriptional deficits, as well as an amelioration of rearing deficits [185]

Table 3. Cont.

	In vitro (cell free)	Cellular model	Animal model
Quercetin	∗ ↓ aSyn fibrillization [146]	∗ ↓ Aβ induced cytotoxicity, protein oxidation, lipid peroxidation and apoptosis in cultured neurons [186] ∗ Protected cells from oxidative insults, IL-1β and PD related toxins [187] ∗ Control immune response via modulation of IL-1β and TNF-α and reduced the proliferation of peripheral blood mononuclear cells isolated from MS patients [188].	Neurodegeneration rodent models: ∗ ↑ memory and synaptic plasticity upon chronic lead exposure [189] ∗ Protection against colchicine-induced cognitive impairment [190] ∗ Improved motor function in a model of acute spinal cord injury [191] AD rodent model: ∗ ↑ Performance on learning and spatial memory tasks and greater risk assessment behaviour [192] ∗ ↓ Extracellular β-amyloidosis, tauopathy, astrogliosis and microgliosis in the hippocampus and the amygdala [192] ∗ ↓ Plaque burden and mitochondrial dysfunction (↑AMPK activity) and ↑ cognitive impairment [193] PD rodent model: ∗ Neuroprotective by inducing antioxidant defences and ATPases [194] Cerebral ischemia rodent model: ∗ ↓ Lesion [195] ∗ ↓ Hippocampal neuronal death [196] ∗ ↓Apoptosis (activation of BDNF-TrkB-PI3K/Akt signalling pathway) [197]
Rosmarinic acid	∗ Scavenging peroxynitrite [101]	∗ Protects neurons from oxidative stress [198, 199]	AD and ALS rodent model: ∗ Alleviated memory impairment, delayed disease onset and ↑lifespan [132, 200]
Hesperetin		∗ Cytoprotective effects in mouse primary neurons [135] ∗ Cytoprotective in a cell model of PD induced by rotenone [201]	AD rodent model: ∗ Restore deficit in non-cognitive nesting ability and social interaction; attenuation on β-amyloid deposition, plaque associated APP expression, microglial activation and TGF-β immunoreactivity [202]

By far, the most explored ND in the aim of (poly)phenols neuroprotection is AD. The majority of the mammalian cell models employed in (poly)phenol research in AD takes advantage of cell lines or primary cultures, being neuroblastoma cell lines the most frequently used. In such models, disease phenotype is induced by the addition of Aβ peptide or toxic Aβ oligomers to the medium[203], by the overexpression of Tau protein, or even by transfection of cells with the amyloid precursor protein (APP)[204]. In these studies, (poly)phenols have been described to protect from Aβ toxicity[205], favouring the formation of stable protofibrils, reversing the rise of ER markers as well as tau

phosphorylation[206], increasing the cytosolic levels of calcium that leads to AMPK activation[207], and diminishing extracellular accumulation of Aβ[207].

Protection from neurotoxicity induced by Aβ was already observed for green tea extracts, in primary rat cortical neurons[103]; blueberry anthocyanin-rich extract, in Neuro2a cells[122]; and Korean black soybeans anthocyanin-rich extract, in the hippocampal HT22 cell line[127]. Other mechanisms impaired by Aβ were also described to be ameliorated by some extracts. For example, blueberry anthocyanin-rich extract increase microglial Aβ clearance, inhibit its fibrillation and suppress microglial activation in murine cell cultures[124]. Similarly, the rescue of αSyn aggregation toxicity was described for *C. album* (poly)phenols extract in human neuroglioma cells[208].

Similarly to AD, mammalian cell models available for studying PD are quite diverse, ranging from cell lines to primary cells. PD is usually associated with an increase of oxidative stress and mitochondrial dysfunction[209]. Due to their pleotropic activities, the search for compounds targeting PD has identified many phenolics as potential effectors redox and mitochondria homeostasis. The models used are mostly models of mitochondrial dysfunction induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine/ 1-methyl-4-phenylpyridinium (MPTP/MPP^+)[160], 6-hydroxidopamine (6-OHDA)[210], paraquat[211] or rotenone injury[201] and several studies used cell lines, such PC12, N27 or SH-SY5Y as PD models to evaluate the potential of phenolics compounds. Notwithstanding, models based on αSyn aggregation are less explored in the field of (poly)phenols, comparatively with Aβ-based models, for instance. Nevertheless, since αSyn aggregation is an important pathological process in PD, αSyn-based cell models should be further improved to better understand the effect of (poly)phenols as potential molecules against PD.

The potential of resveratrol in NDs prevention has been extensively exploited. It was shown to reduce Aβ mediated accumulation of ROS and apoptosis in cell models[167], being its protection related with the modulation of NF-κB and SIRT1 pathways[167-169]. In yeast, resveratrol mimics caloric restriction, described to slow the pace of ageing, by stimulating Sir2 (the homologue of SIRT1), increasing DNA stability and extending life span by 70%, being therefore associated with a mitigation of age-related diseases,

including neurodegeneration[212]. Microbial-derived phenolic metabolites found in blood after wine consumption, 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, and salicylic β-D-*O*-glucuronide have shown to be neuroprotective by significantly decreasing mitogen-activated protein kinase (MAPK) p38 and ERK 1/2 activation, as well as in caspase 3 activity in SH-SY5Y cells[154]. The neuroprotective effect of selected bioavailable (poly)phenol derived metabolites (namely 3,4-dihydroxyphenylacetic acid, 3,4-hydroxyphenylpropionic acid, gallic acid, ellagic acid and urolithins) against oxidative stress in SH-SY5Y cells was also already reported, being involved with the prevention of neuronal apoptosis[213]. In another study, the secretion of Aβ from neurons and associated damage seems to be attenuated by gallic acid treatment[214].

Ellagitannin-derived metabolites (urolithins and pyrogallol) have shown to be effective antiglycative agents, whereas chlorogenic acid-derived metabolites (dihydrocaffeic acid, dihydroferulic acid and feruloylglycine), generated *in vivo* in the colon, proven to be effective in combination in protecting neuronal cells, being able to counteract key features of diabetic complications, *i.e.* protein glycation and neurodegeneration[152].

Curcumin has also been pointed as a (poly)phenol with a plethora of protective activities[180, 215] including MPTP protective activity[181]. Using rat primary hippocampal cultures, it was show that tetrahydrocurcumin, a metabolite of curcumin, presented protective effects against oligomeric Aβ induced toxicity[216].

The flavonoid quercetin is also among the well-characterized (poly)phenols in terms of biological activity [reviewed in [160, 187]]. Pre-treatment of primary hippocampal cultures with quercetin significantly attenuated Aβ-induced cytotoxicity, protein oxidation, lipid peroxidation and apoptosis[186]. Moreover, the brain-available metabolite of quercetin, quercetin-3-O-glucuronide, also significantly inhibited the generation of Aβ(1–40) peptides from Tg2576 primary neuron cultures[149],

5-(3', 5'-dihydroxyphenyl)-γ-valerolactone, the main ring-fission metabolite of EGCG, showed slightly higher BBB permeability than EGCG and presented neuritogenic activity in SH-SY5Y cells, suggesting a possible role of this metabolite in brain neurogenesis[217].

Chlorogenic acid, a (poly)phenol present in coffee, was shown to protect cortical primary neurons against glutamate neurotoxicity, with potential for ischemic stroke treatment[218].

The 3-O-caffeoylquinic acid reduced neuroinflammation by reducing microglia mediated

ROS production and neuronal excitotoxicity[219], whereas hesperetin, a flavanone glycoside abundant in citrus fruits, have also revealed neurprotective effects in mouse primary neurons[135] and in a rotenone-induced apoptosis human neuroblastoma SK-N-SH cells, considered a cellular model of PD[201]. Other study reported that less representative flavonoids (chrysin, puerarin, naringenin, genestein) presented neuroprotective activity in mesencephalic cultures challenged with MPP^+, another model of PD[160].

The studies presented in **Table 1** have in common the use of complex mixtures of compounds of dietary origin and the studies presented in **Table 3** have in common the use of aglycones of parent (poly)phenols. However, they do not consider (poly)phenols' metabolism and therefore they could only be considered in a pharmacological perspective with controlled target delivery to the CNS. In fact, the described effects seen in these cellular assays could be completely altered by human metabolism and therefore metabolites mechanism of action should be considered. Blackberry extracts submitted to a simulated gastro-intestinal digestion revealed that neuronal protection to oxidative insult can be unrelated to the modulation of reactive oxygen species (ROS) and glutathione (GSH) levels, suggesting a pre-conditioning effect by the induction of caspase activity[220, 221]. Moreover, bioaccessible raspberry metabolites resulting from the *in vitro* digestion of raspberry extract significantly inhibited microglial pro-inflammatory activation by LPS through the inhibition of Iba1 expression, TNF-α release and NO production[222]. Such studies, taking advantage of (poly)phenol components resulting from *in vitro* digestion, present a step forward in terms of considering human metabolism of (poly)phenols and comprise a more physiologically-relevant approach.

Cellular studies taking into consideration (poly)phenols metabolism and biological activity against neurodegeneration (**Table 4**), using physiologically-relevant concentrations and relevant resident time, mark a mentality shift and the beginning of a new era in phytochemical research. Such research work, however, is in its infancy and there is still a lot of questions that remain to be answered.

Table 4. Neuroprotective evidences (using cellular models) for some bioavailable (polyphenol metabolites.

(Poly)phenol metabolite(s)	Cell model	Neuroprotective evidence	Reference
epicathechin epicathechin glucuronide 3'-O-methyl epicatechin	Primary cultures of mouse cortical neurons + H_2O_2	Pre-treatment with epicathechin glucuronide exibited no significant protection, but both epicathechin and 3'-O-methyl epicatechin were neuroprotective	Spencer *et al.* 2001[223]
epicatechin 3'-O-methyl epicatechin	Primary cultures of mouse striatal neurons + oxLDL	A major in vivo metabolite of epicatechin, 3'-O-methyl-epicatechin was as effective as epicatechin in protecting neurons from oxidized low-density lipoprotein (oxLDL) as the oxidative insult	Schroeter *et al.* 2001[224]
hesperetin hesperetin-7-O-β-D-glucuronide nitro-hesperetin	Primary cultures of mouse cortical neurons + H_2O_2	Whilst hesperetin glucuronide failed to exert protection, both hesperetin and 5-nitro-hesperetin were effective at preventing neuronal apoptosis via a mechanism involving the activation/phosphorylation of both Akt/protein kinase B and extracellular signal-regulated kinase 1 and 2 (ERK1/2)	Vauzour *et al.* 2007[225]
urolithin A urolithin B urolithin C urolithin D 8-O-methylurolithin A 8,9-di-O-methylurolithin C 8,9-di-O-methylurolithin D punicalagins punicalins ellagic acid gallic acid	Myelomonocytic HL-60 cells + phorbol 12-myristate-13-acetate	The antioxidant activity of urolithins was correlated with the number of hydroxy groups as well as the lipophilicity of the molecule. The most potent antioxidants are urolithins C and D, being higher than the parent ellagic acid and punicalagins. The dihydroxylated urolithin A showed weaker antioxidant activity; however, the potency was within the range of urolithin A plasma concentrations.	Bialonska *et al.* 2009[226]
urolithin A urolithin B pyrogallol dihydrocaffeic acid dihydroferulic acid feruloylglycine 3-hydroxyphenylacetic acid 3,4-dihydroxyphenylacetic acid 3-methoxy-4-hydroxyphenylacetic acid	SK-N-MC cells + 2,3-dimethoxy-1,4-naphthoquinone	Chlorogenic acid-derived catabolites (dihydrocaffeic acid, dihydroferulic acid and feruloylglycine) were most effective in combination in protecting neuronal cells from oxidative stress.	Verzelloni *et al.* 2011[152]
tetrahydrocurcumin	Primary hippocampal neuron cultures + oligomeric Aβ	Tetrahydrocurcumin protects hippocampal cells against Aβ-induced toxicity. Tetrahydrocurcumin may have beneficial effects in Alzheimer's disease and other neurodegenerative diseases that involve oxidative stress and neuronal loss.	Mishra *et al.* 2011[216]
quercetin-3-O-glucuronide malvidin-3-O-glucoside	Primary Tg2576 neuron cultures + Aβ(1-40)	Brain-targeted quercetin-3-O-glucuronide significantly inhibits generation of Aβ(1–40) peptides from Tg2576 primary neuron cultures.	Ho *et al.* 2013[149]

Table 4. Cont.

(Poly)phenol metabolite(s)	Cell model	Neuroprotective evidence	Reference
protocatechuic acid vanillic acid 4-hydroxybenzoic acid benzoic acid-glucuronide benzoic acid-sulfate protocatechuic acid-3-glucuronide protocatechuic acid-4-glucuronide protocatechuic acid-3-sulfate protocatechuic acid-4-sulfate vanillic acid-glucuronide vanillic acid-sulfate isovanillic acid isovanillic acid-glucuronide isovanillic acid-sulfate	THP-1 monocytic cells + LPS	Five metabolites (isovanillic acid, isovanillic acid -glucuronide, benzoic acid-sulfate, protocatechuic acid-3-sulfate, and vanillic acid-glucuronide) reduced LPS-induced TNF-α secretion. Some bioavailable metabolites of flavonoids, individually and in combination, appear more bioactive then their precursor flavonoids, at equivalent concentration	diGesso et al. 2015[153]
astragaloside IV cinnamic acid paeoniflorin gallic acid	SH-SY5Y cells + anisomycin	Gallic acid was able to reduce anisomycin-induced Aβ deposition, suggesting that gallic acid exerted protective effects on SH-SY5Y cells, which may be considered a novel therapeutic strategy in the treatment of patients with AD	Wang et al. 2016[214]
gallic acid ellagic acid 3,4-dihydroxyphenylpropionic acid 3,4-dihydroxyphenylacetic acid trans-resveratrol trans-resveratrol-O-sulfate trans-resveratrol glucuronide dihydroresveratrol dihydroresveratrol-O-sulfate dihydroresveratrol-O-glucuronide urolithins (A, B, C, D) urolithin A 3-O-glucuronide urolithin A 8-O-glucuronide iso-urolithin A iso-urolithin A 3-O-glucuronide iso-urolithin A 9-O-glucuronide	SH-SY5Y cells + H_2O_2	Among the 19 metabolites tested, 3,4-dihydroxyphenylpropionic acid, 3,4-dihydroxyphenylacetic acid, gallic acid, ellagic acid, and urolithins prevented neuronal apoptosis via attenuation of ROS levels, increased REDOX activity, and decreased oxidative stress-induced apoptosis by preventing the caspase-3 activation via the mitochondrial apoptotic pathway in SH-SY5Y cells.	González-Sárrías et al. 2017[213]
pyrogallol 5-(3',5'-dihydroxyphenyl)-γ-valerolactone 5-(3',5'-dihydroxyphenyl)-γ-valerolactone-glucuronide 5-(3',5'-dihydroxyphenyl)-γ-valerolactone-sulfate	SH-SY5Y cells	In human neuroblastoma SH-SY5Y cells, neurite length was significantly prolonged by 5-(3',5'-dihydroxyphenyl)-γ-valerolactone, and the number of neurites was increased significantly by all metabolites examined.	Unno et al. 2017[217]
3,4-Dihydroxyphenylacetic acid 3-(3-hydroxyphenyl) propionic acid 3-(4-hydroxyphenyl) propionic acid 3-hydroxyphenylacetic acid salicylic acid salicylic β-d-O-glucuronide	SH-SY5Y cells + SIN-1	Cell pretreatment with microbial metabolites found in blood after wine consumption, 3,4-dihydroxyphenylacetic, 3-hydroxyphenylacetic acids and salicylic β-d-O-glucuronide, at physiologically concentrations (0.1-10 μM) resulted in increased cell viability versus SIN-1 control group	Esteban-Fernández et al. 2017[154]

Table 4. Cont.

(Poly)phenol metabolite(s)	Cell model	Neuroprotective evidence	Reference
urolithin A urolithin B methyl-urolithin A methyl-urolithin B	BV2 microglial cell + LPS BV2 microglial cell + H_2O_2 SH-SY5Y cells + H_2O_2 BV2 microglial cell + LPS (conditioned medium) applied to SH-SY5Y cells	Urolithins decreased media levels of nitric oxide, interleukin 6 (IL-6), prostaglandin E2, and tumor necrosis factor alpha from LPS-BV2 microglia. In the co-culture cell model, media from LPS-BV2 cells treated with urolithins preserved SH-SY5Y cell viability greater than media from cells treated without urolithins. Urolithins mitigated apoptosis and caspase 3/7 and 9 release from H_2O_2-induced oxidative stress of BV2 and SH-SY5Y cells.	DaSilva et al. 2017[227]

Considerations regarding cellular models in (poly)phenols research

Cellular models are indispensable to understand the molecular mechanisms of (poly)phenols and (poly)phenol metabolites and how they can affect cellular homeostasis. However, current studies take conclusions from 2D systems and using cell lines as disease models, which still present some limitations for a real physiological answers. Therefore the current trend of cellular models to study NDs for drug development is already moving away from mammalian cell lines, converging into more complex cell systems. Advances in reprogramming technology have allowed the generation of human neurons derived from fibroblasts of disease patients. These cells harbour a disease genetic background allowing the recapitulation of AD or PD pathogenesis *in vitro* when differentiated into mature neurons[228-230]. Such models, however, still present limitations mainly associate with the limitation of reconstituting ND conditions *in vitro* over a short time course while the pathogenic changes of NDs progress slowly. In addition, differentiation and maturation of human neural stem cells occurs over a span of months, and it may not be enough to establish the aged brain conditions under which patients develop robust neurodegenerative pathologies[231]. Studies have shown that 3D conditions recapitulate more closely the in vivo scenario and can accelerate neuronal differentiation and neural network formation[232-234]. Neurons grown in a 2D environment do not have the support for vertical growth, which can lead to inadvertent polarity instead of a more precise neuronal phenotype[235]. Additionally, in 2D, the space between cells does not recapitulate the in vivo situation: 2D models tend

to have reduced connections between neurons, and synaptic distances are bigger than physiological. Neurons grown in a 3D environment are able to express neuronal markers more accurately, as compared to neurons grown in a monolayer, with elevated mature neuronal marker expression[236, 237]. Moreover, in 2D cell cultures, the secreted Aβ species from fibroblasts of AD patients diffuse into the cell culture media, which will be removed during regular media changes. Such phenomenon may explain why it is difficult to detect Aβ aggregation in a 2D cell culture, for instance. In this sense, 3D cultures provide a local environment that promotes aggregation of β-amyloid, which can trigger pathogenic cascades[237], being a more suitable model for studying disease pathology. 3D culture conditions, as compared with 2D, present additional advantages in recapitulating ND conditions, being able to be applied in large scale for high-throughput screening for novel therapeutic targets, or even for validation during the initial stages of drug discovery that are not feasible in the current animal models[237-239]. The development of increasingly robust advanced models systems is a fast-growing field owing the great demand by the pharmaceutical industry. In the future, (poly)phenol research can benefit from their utilization to accelerate the discovery of bioactive molecules with potential application in the therapeutics of neurodegenerative conditions.

Nevertheless, cellular models are indispensable to understand the molecular mechanisms of (poly)phenols and how they affect cellular homeostasis. Currently, there are already a lot of evidences for pure parent (poly)phenols mode of action regarding their potential against neurodegeneration. However, the presence of these compounds in neuronal tissues can only be considered by pharmacological approaches. There is still a considerable gap in knowledge regarding (poly)phenol metabolites potential taking advantage of cell models. These nutritionally-relevant metabolites are the ones that circulate in higher percentage and that can comprise the true effectors against neurodegeneration. Yet, in order to design nutritional or pharmaceutical approaches using (poly)phenols, it is mandatory to translate their benefits into animal models, and to study their effects at the level of the whole organism.

3.3. *In vivo* animal assays

Most of the evidences for neuroprotection in animal models come from studies where foods or plant extracts are given orally to animals. Those are defined as nutritional (nutraceutical) interventions since (poly)phenols can cross/interact with the gastro-intestinal barrier and be metabolized as dietary compounds. Only encapsulated compounds, intravenous or other forms of delivery can be considered in a pharmacological perspective.

Accumulating evidence of (poly)phenol potential *in vivo* reinforces the *in vitro* (cell free) and cellular models conclusions. Intake of berries such as pomegranate, strawberry, blueberry and blackberry ameliorated several aspects of memory and learning[240], delayed age-related motor and cognitive behavioural deficits in rodent models[130, 132, 241]. Oral administration of quercetin protected against colchicine-induced memory impairment and oxidative damage in rats[190]. In PD *in vivo* models, quercetin showed to be neuroprotective by inducing antioxidant defences and ATPases[194].

Several attempts were done with a pharmacological approach, by direct injection of bioactive (poly)phenols to the organism with the aim to circumvent its metabolism and improving their efficacy. Resveratrol was tested by intraperitoneal injection on a 6-hydroxydopamine (6-OHDA)-induced PD in rats and revealed attenuation of oxidative stress, lipid peroxidation, and protein carbonyl content[173]. Notably, curcumin when injected in the tail vein of an AD rodent model, was able to cross the BBB, target and disrupt existing plaques[184]; moreover, it also reduced Aβ plaques, oxidized proteins and IL-1β in AD transgenic mice[144]. Intraperitoneal administration of quercetin improved motor function in a model of acute spinal cord injury[191], reduced ischemic lesion[195], as well as hippocampal neuronal death[196], and also cell apoptosis in a rat model of cerebral ischemia[197]. By the same route, quercetin improved memory and synaptic plasticity upon chronic lead exposure in rats[189].

Despite of the importance of this studies in a pharmacological perspective, the understanding of the true bioavailable (poly)phenol metabolites effects should be better clarified. Only having such information we may be able to translate the nutritional value of (poly)phenol-rich foods towards human health. Some authors have already started to take such considerations into account. A study have shown that both curcumin and its

metabolite tetrahydrocurcumin exerted neuroprotection against MPTP induced neurotoxicity in mice[242]. Two brain-available polyphenol metabolites, quercetin-3-O-glucuronide and malvidin-3-O-glucoside, significantly attenuated sleep deprivation-induced cognitive impairment in a mouse model of acute sleep deprivation[243].

Table 5. Neuroprotective evidences (*in vivo*) for some bioavailable (polyphenol metabolites.

(Poly)phenol metabolite(s)	Animal model	Neuroprotective evidences	Reference
curcumin tetrahydrocurcumin	Mice + MPTP	Curcumin and tetrahydrocurcumin reversed the MPTP induced depletion of dopamine and DOPAC which may in part be due to inhibition of MAO-B activity, exerting neuroprotection against MPTP induced neurotoxicity	Rajeswari *et al.* 2008[242]
genistein equol	Male and ovariectomized female Sprague-Dawley rats	Genistein and equol significantly reduced infarct size in both sexes. Neuroprotection in ovariectomized female rats was accompanied by a decrease in NAD(P)H oxidase activity and superoxide levels in the brain. In addition, equol reduced plasma thiobarbituric acid reactive substances, and neurological deficits	Ma *et al.* 2010[244]
equol	Rats + cerebral ischemia/reperfusion injury	Equol decreased the mortality, neurological deficit, brain histological damage, infarct volume, serum lactate dehydrogenase activity and malondialdehyde content in a dose-dependent manner in rats	Wei *et al.* 2014[245]
quercetin-3-O-glucuronide malvidin-3-O-glucoside	Mice model of acute sleep deprivation	Quercetin-3-O-glucuronide and malvidin-3-O-glucoside, significantly attenuated sleep deprivation-induced cognitive impairment in a mouse model of acute sleep deprivation	Zhao *et al.* 2015[243]
punicagalin punicalin ellagic acid gallic acid urolithin A urolithin B methyl-urolithin A methyl-urolithin B	*Caenorhabditis elegans* + Aβ(1-42)	Methyl-urolithin B but not pomegranate predominant ellagitannins had a protective effect in *Caenorhabditis elegans* post induction of Aβ(1−42)-induced neurotoxicity and paralysis	Yuan et al. 2016[150]
ellagic acid urolithin A urolithin B urolithin C urolithin D	*Caenorhabditis elegans*	Urolithins extend lifespan and improve fitness in *C. elegans.* Treatment with ellagic acid had no effect on lifespan. Urolithin A exerts its beneficial effects through the induction of mitophagy	Ryu *et al.* 2016[246]

An integrated view of *in vitro*, cellular and animal model studies either with food or plant extracts or pure compounds, parent (polyphenols and (poly)phenol metabolites (**Tables 1-5**) reveals a complete panorama of knowledge gathered for (poly)phenols, and clearly reinforces their neuroprotective potential. Although human trials are more reliable and

the closest to the real scenario, they are time and cost consuming. The animal and cellular models have constituted unparalleled tools to dissect mechanisms of action, and for genetic and compound screening assays. On the other hand, the *in vitro* assays are crucial to understand the interaction of (poly)phenols with proteins, metals or other small molecules, by excluding cell components interference and serving as a fundamental tool to tune the overall picture revealed by cell studies. Nevertheless, the importance of (poly)phenols and (poly)phenol-rich foods against relevant aspects of neurodegeneration should be properly addressed to provide relevant tools to validate the aspects observed using the aforementioned models.

3.4. Human trials

Human clinical trials to ascertain the impact of (poly)phenols in NDs and ageing are still very scarce. Although allusive of a protective role in neurodegeneration, there are still too many fragmented evidences to build an integrated picture based on the mechanistic studies.

Concerning green tea extracts, human studies revealed the increase of brain activity in the dorsolateral prefrontal cortex, an area involved in memory processing[247]. Recently, consumption of green and black tea by healthy volunteers increased the brain theta waves, suggesting a role in cognitive function, specifically alertness and attention[248]. Moreover, EGCG reduced cognitive deficits in a pilot study with Down syndrome individuals, with effects on memory recognition, working memory and quality of life[249].

In an intervention study, blueberry juice supplementation improved memory in older adults[59]. Also, supplementation with Concord grape juice enhanced neurocognitive function in older adults with pre-described mild cognitive impairments, which was supported by studies on brain activity using functional magnetic resonance imaging[250].

There are several studies examining flavanols and anthocyanins neuroprotective properties in humans but little investigation into the flavonoid subclass known as flavanones. This is an incredible gap in knowledge once orange juice, a rich source of flavanones, is one of the most-commonly consumed juices throughout the world. A recent study evaluated the consumption of flavanone-rich orange juice over 8 weeks and concluded that there was an association with benefits for global cognitive function

in healthy older adults, in comparison to the consumption of a low-flavanone control juice[251]. Moreover, the same authors observed that ingestion of a flavonoid-rich orange juice promoted acute cognitive benefits over 6 h in healthy middle-aged adults[252].

A recent study examined the effects of curcumin on cognition and mood in a healthy older population. Working memory and mood were significantly improved following 4 weeks treatment, confirming the potential psychological and cognitive benefits of curcumin in an older population[253].

On the other hand, a contradictory study in a large community sample, with a 12-week supplementation of quercetin, provided evidence that quercetin may not have an ergogenic effect on neurocognitive functioning[254], consistent with a growing body of literature raising concerns about the generalization of findings from *in vitro* and animal quercetin research to human populations.

Two recent studies have reinforced the impact of diets rich in berries for cognition improvement in older adults: a chronic blueberry supplementation improved brain perfusion, task-related activation, and cognitive function in healthy older adults[62], and in a double-blind, placebo controlled trial, the addition of blueberry to the diets improved some specific aspects related with cognition[63].

Some human studies have been performed for dietary formulations that include mixtures of (poly)phenols or (poly)phenols with other compounds, highlighting possible synergies between constituents. For instance, in a double-blind clinical trial with older adults[255], the intake of a pill-based nutraceutical that contained a proprietary formulation of blueberry, green tea, carnosine, vitamin D3 and biotin, resulted in significantly increased processing speed. Also, in healthy overweight older individuals, a daily intake of a formulation containing resveratrol and quercetin significantly improved memory performance[256].

The evaluation of (poly)phenol metabolites effects in humans could be more difficult. Although these metabolites are produced in the human gut, the administration of synthetic (poly)phenol metabolites to humans can raise safety concerns since these are not found as such (at least in relevant concentrations) in foodstuffs, and therefore they cannot be considered as dietary compounds. In this regard, the toxicological evaluation of (poly)phenol metabolites in animal models should be properly assessed in advance.

Nevertheless, for (poly)phenols and their circulating metabolites reach the brain and be able to exert neuroprotective actions, they have to be able to cross to and/or interact with the BBB. An increasing body of evidence regarding this subject has been growing and the next section will discuss the latest advances regarding it.

4. (POLY)PHENOLS AND RESTRAINS: INTERACTION WITH BARRIERS AND BEYOND

The extensive metabolism to which dietary (poly)phenols are submitted once ingested, either in the intestine, liver and or in cells, leads to the arise of a broad range of (poly)phenol derivatives. Despite accumulating evidence concerning dietary (poly)phenols aglycones and their metabolites neuroprotection (**Tables 1**-**5**), and assuming the effective transport/distribution/delivery of dietary (poly)phenol metabolites to target tissues, we also need to consider the existence of other barriers that must be crossed by these (poly)phenols metabolites or, at least, be able to alter the surrounding environment in order to induce responses in the target organs.

4.1. Blood-brain barrier

The existence of three layers of barrier at the CNS limits and regulates molecular exchanges between the blood and the neuronal tissue, or its fluidic spaces: i) the BBB, majorly constituted by cerebromicrovascular endothelial cells, located between the blood and the brain interstitial fluid; ii) the choroid plexus epithelium, located between the blood and ventricular cerebrospinal fluid; iii) and the arachnoid epithelium, located between the blood and subarachnoid cerebrospinal fluid[257]. In particular, the choroid plexus is responsible for the transport of vitamins, small peptides, amino acids, inorganic ions and hormones to the cerebrospinal fluid[258]. Among the different barriers of the CNS, the BBB exerts the highest control over the close microenvironment of brain cells, since its surface area is 5000 times larger than the blood-cerebrospinal fluid barrier, having a dominant role in providing nutrients for the brain, as well as controlling metabolites, like (poly)phenols, accessibility to neuronal cells [259].

The BBB is a dynamically selective and complex interface, protecting the CNS from toxic compounds and pathogens, acting as a border between the periphery and the brain[260]. Besides the microvascular endothelial cells that comprise the anatomical basis of the BBB, it is surrounded and interacts with perycites, astrocytic endfeet, microglia and neurons, which constitute the neurovascular unit (**Fig. 4**) [257, 259, 261]. The neurovascular unit complexity, together with the tight control exerted by the brain endothelium due to the presence of intracellular tight junctions, lack of fenestrations, low pinocytic activity and efflux pumps, controls the passage of the smallest polar molecules and macromolecules[259, 262, 263] (**Fig. 4**). (Poly)phenols are no exception and in order for them or their metabolites to access the brain, they must cross a tightly regulated, selectively permeable endothelial layer. As a growing number of biological effects have been attributed to these molecules in the past few years, attention to their role as modulators of the transport capacity of epithelial barriers has also received great attention.

Youdim and co-workers have attempted to demonstrate (poly)phenols permeation through the BBB[264, 265]. They suggested that (poly)phenols transmembrane diffusion *in vitro* is related with its lipophilicity, where less polar derivatives (e.g. methylated derivatives) are capable of higher brain uptake than more polar metabolites (e.g. sulphated and glucuronides). Accumulating evidence of (poly)phenols uptake by the BBB reinforces their putative potential in a neurological context. Nevertheless, it is not yet totally clear whether the primary route by which (poly)phenols cross the BBB is simple diffusion or carrier-mediated transport[266]. To date, only a few compounds from each flavonoid subclasses have been studied, and there is limited knowledge on the effects of flavonoid structure on their bioavailability at the brain level. Therefore, the true mechanisms by which flavonoids and their circulating metabolites interact with the BBB remain a hot topic in neuroscience research.

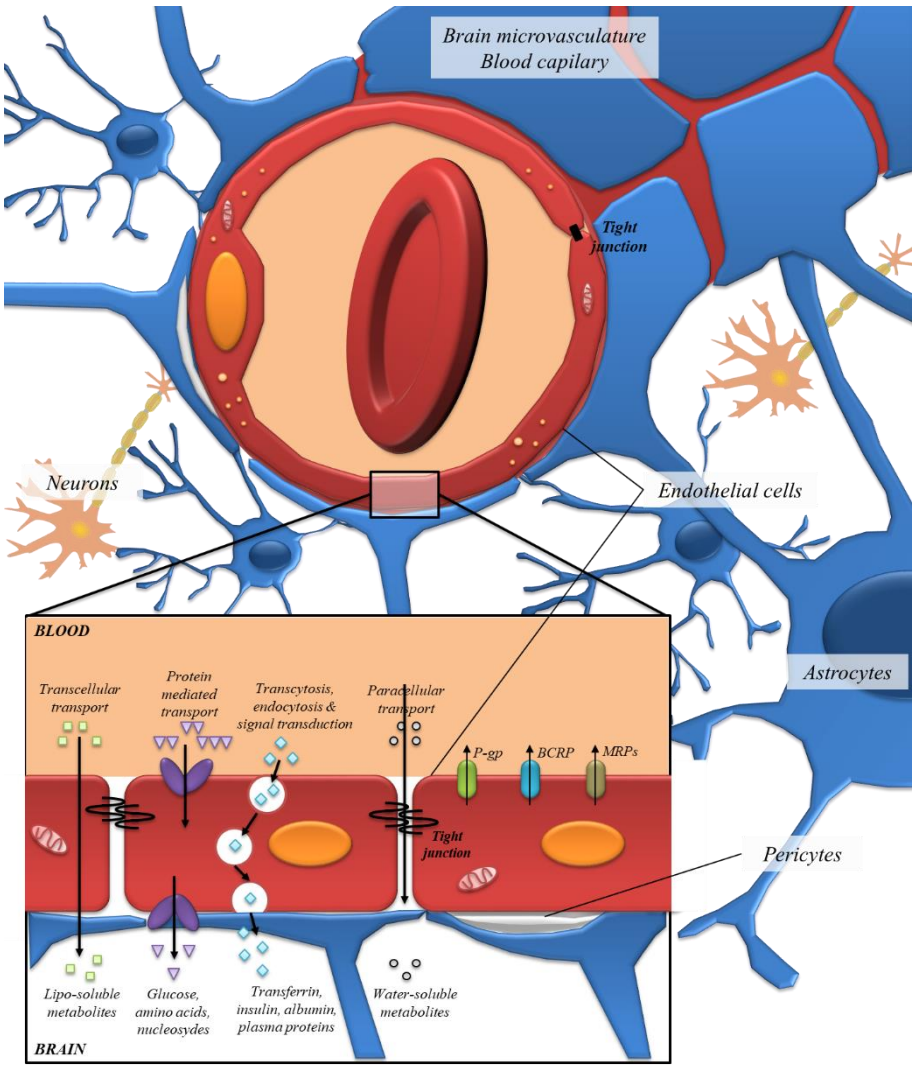

Fig 4. The neurovascular unit. The BBB is composed by endothelial cells, astrocytes and perycites, where endothelial cells form a boundary between the blood and the CNS. The flux of nutrients and metabolites from the blood to the CNS is regulated by the BBB, which controls their availability by transport systems. Transport across the BBB occurs via several pathways: lipid-mediated diffusion (transcellular transport), paracellular diffusion, carrier-mediated transport through proteins, receptor-mediated transcytosis and absorptive-mediated transcytosis. Besides these pathways, active efflux transport through ABC transporters such as P-gp, BCRP and MRPs family also takes place, in order to prevent the entry and accumulation of harmful substances to the brain.

Considering (poly)phenol and their metabolites permeability across the BBB, the existence of different specialized transporters in the plasma membranes of luminal (blood) and abluminal (brain) sides of the endothelial cells should be taken into account.

The endothelial cells permeability to (poly)phenols is particularly influenced by the expression profile, functionality and precise location/orientation of efflux transporters, solute carriers and organic-anionic transporters[267]. Nevertheless, there are strong evidences of the effective capacity of (poly)phenols to reach the brain and exert neuroprotection, and it is an ever-growing field of investigation (**Table 6**).

In terms of uptake, there is evidence suggesting that transport mediated by glucose transporter 1 (GLUT1), organic anion transporters (OATs) and organic anion-transporting polypeptide (OATP1A2) may affect the distribution of flavonoid glucuronides into the brain, in a similar fashion to other glucuronidated metabolites[268, 269]. In fact, the presence of these glucuronidated metabolites can be partially derived, due to the high expression of both GLUT1 and OATP1A2 on both luminal and abluminal membrane of endothelial cells. In a recent study, the authors successfully localized quercetin-3-O-glucuronide, a major phase-II metabolite of quercetin, in human brain tissue, namely at epithelial cells of the choroid plexus and also in macrophages[270].

Microdialysis sampling in rats also showed that (+)-catechin and (-)-epicatechin could pass through the BBB[271]. In fact, several (poly)phenols have been identified in different brain regions of the rat[272, 273] and pig[274, 275], usually accumulating in a nonregion-specific manner[276]. Several animal studies also indicate that (poly)phenols are able to cross the BBB and co-localize within the brain tissues independently of their route of administration: epigallocatechin gallate[277], epicatechin[278], and anthocyanins[273, 279] were detected in the brain after oral administration; naringenin and its glucuronide were identified in the cerebral cortex, after intravenous administration[280], and intravenously administered hesperetin was also detected in the brain, especially in the striatum[281].

Another study has examined the permeability of flavonoids and their known circulating metabolites across an *in vitro* model of the BBB[264]. Hesperetin, naringenin, and their respective *in vivo* glucuronides, as well as the anthocyanins cyanidin-3-rutinoside and pelargonidin-3-glucoside, all showed measurable permeability. In another study, both catechin and epicatechin could cross a cellular model of BBB in a time-dependent and stereo-selective manner, with epicatechin presenting a substantially higher transport than catechin[282]. *In vitro* transmembrane transport of different flavonoids (flavonols, flavan-3-ols and anthocyanins) and some of their methylated and glucuronidated

metabolites was also observed, where in most cases the metabolites exhibited higher transport efficiency than their parent compounds[283]. All these collected data strongly suggest the effective uptake of dietary (poly)phenols and their metabolites through the BBB endothelium, reinforcing their ultimate neuroprotective potential.

(Poly)phenols as modulators of membrane transport

Importantly, (poly)phenols have also shown to be able to modulate the activity of some ATP-binding cassette transporters (ABC transporters). The ABC transporters are efflux pumps responsible for controlling the bioavailability of many xenobiotics, being vastly present in the epithelia of the gut, placenta and BBB, as well as in cancer cells. Cancer cells tend to overexpress these ABC transporters, which confers them bigger resistance to chemotherapy[284].

Several studies have demonstrated inhibition of ABC transporters by flavonoids (**Table 6**). The interaction between flavonoids and ABC-transporters could be advantageous for poorly absorbed drugs; otherwise, this interaction could also lead to drug intoxication, particularly in the context of drugs with a narrow therapeutic window. Additionally, some flavonoids are themselves substrates of the most pharmacologically relevant ABC transporters: P-gp, multidrug resistant protein type 2 (MRP2) and breast cancer resistant protein (BCRP)[285].

For example, it was shown that extracts from St John's Wort upregulate the expression of P-gp at intestinal level, which could ultimately reduce the bioavailability of substrate pharmaceuticals[286]. On the other hand, diterpenes, triterpenes and carotenoids, naturally occurring lipophilic phytochemicals, were able to inhibit human P-gp *in vitro* at a low range, while other combinations had a synergistic activity[151, 287]. Quercetin, hypericin and kaempferol were shown to promote the cellular uptake of ritonavir on P-gp overexpressing cells[288]. Besides, both *in vitro* and *in vivo* assays (only with short-term exposure) with these (poly)phenols inhibited the action of efflux pumps and increased substrate bioavailability; *in vivo* assays of chronic exposure boosted the expression of P-gp and lead to a reducing bioavailability of substrate drugs[289, 290].

Besides their modulation of ABC transporter function, (poly)phenols can also be substrates of these transporters, a property that can dramatically limit their bioavailability. Youdim and co-workers have showed the limitations that quercetin has to BBB transposition: when co-administrated with a P-gp or BCRP inhibitor, this (poly)phenol was able to enter epithelial cells of the BBB, possibly by passive diffusion due to its hydrophobicity, but was then specifically exported by the BCRP [264]. Such phenomenon explains, at least in some extent, why quercetin and resveratrol presented very limited bioavailability, despite the amount ingested[291, 292].

Taking all this information into account, it could be interesting to explore the combination of an inhibitor of the ABC transporters and a beneficial (poly)phenol, in order to increase the accumulation of such (poly)phenol in the target region and potentiate its efficiency. The co-administration of a quercetin-rich product and an inhibitor of BCRP may increase quercetin's access into the brain and then unleash novel bioactivities from this (poly)phenol[293]. In addition, if the inhibitors of ABC transporters themselves are orally bioavailable, they may assist (poly)phenols transport through the BBB[265].

Importantly to notice, most of what is known nowadays regarding (poly)phenols interaction/modulation of ABC transporters is essentially about dietary (poly)phenol aglycones effects and not to their circulating metabolites. As such, it is imperative to evaluate in the future the interactions that could be established between transporters and bioavailable (poly)phenols metabolites which chemically diverge from their parent compounds. As such, we may anticipate differences regarding interactions and/or modulation of ABC transporters activity, not directly translatable from what is currently known from their parent counterparts.

Table 6. Evidences of (poly)phenol transport at the BBB, concerning interaction studies with ABC efflux transporters expressed in endothelial cells at the BBB. P-gp - P-glycoprotein, MRP - multidrug resistant protein, BCRP - breast cancer resistant protein, BMEC - brain microvascular endothelial cells, TR-rats - transport deficient rats, N. D. – not described.

(Poly)phenol	Evidence of BBB penetration	Efflux transporter interaction	Experimental setup	
Apigenin	Yes [294]	P-gp inhibitor	Rat BMEC	[294]
		P-gp inhibitor	CCRF-CEM, CEM/ADR5000 leukemia cells	[295]
		BCRP inhibitor	MDA-MB-231-BCRP cells	[295]
Catechin / epicatechin	Yes [282]	P-gp activator	NIH-3T3-G185 cells	[113]
		MRP2 substrate	Caco-2 cells	[296]
		MRP1/MRP2 substrate	MDCKII/MRP1 cells & MDCKII/MRP2 cells	[297]
Chrysin	N. D.	P-gp inhibitor	Mouse BMEC	[298]
		MRP2 substrate	Caco-2 cells	[299]
		BCRP inhibitor	MCF-7 MX100 cells	[300]
Curcumin	Yes [184]	P-gp inhibitor	MCF-7 cells	[301]
		BCRP inhibitor	Rat brain capillaries	[302]
Fisetin	Yes [303]	MRPs inhibitor	Caco-2 cells	[304]
Genistein	Yes [305]	P-gp inhibitor	MCF7/BC19-3, rats	[306, 307]
		MRP2 inhibitor	TR-rats	[308]
		BCRP inhibitor	K562/BCRP cells	[309]
Hesperitin	Yes [264]	P-gp inhibitor	Mouse BMEC	[298]
		BCRP inhibitor	ABCG2 over-expressing cells	[310]
Kaempferol	Yes [294]	P-gp inhibitor	Mouse BMEC	[298]
		MRPs inhibitor	Human glioblastoma cell line T98G	[311]
		BCRP inhibitor	MDCK/Bcrp1 cells	[312]
Myricetin	Yes [313]	P-gp inhibitor	MCF-7/ADR cells	[314]
		BCRP inhibitor	HEK293/ABCG2 cells	[284]
Naringenin	Yes [291, 315]	P-gp inhibitor	Mouse BMEC	[298]
		MRPs inhibitor	HEK293/ABCG2 cells	[284]
Quercetin	Yes [265]	P-gp inhibitor	Mouse BMEC	[298]
		MRPs inhibitor	HEK293/MRP1, HEK/MRP4, HEK/MRP5 cells	[316]
		BCRP inhibitor	ABCG2 over-expressing cells	[310]
Resveratrol	Yes [207]	P-gp inhibitor	MCF-7/ADR cells	[317]
		BCRP inhibitor	ABCG2 over-expressing cells	[310]
Rutin	Yes [294]	P-gp inhibitor	Rat BMEC	[294]

4.2. Beyond barriers

In the light of today's knowledge, novel mechanisms of action for (poly)phenols are proposed far beyond the classical direct antioxidant radical scavenging power. In fact,

(poly)phenol concentration at cerebrospinal fluid rarely exceeds 1-5 μmol. Therefore, it is highly improbable that (poly)phenol intracellular concentrations in neurons and glial cells would exceed the micromolar-nanomolar range, which is not enough to exert significant direct antioxidant effects. Nevertheless, such concentrations are in fact beneficial, pointing out that (poly)phenols and their metabolites modulate other cellular parameters and pathways at the CNS level, even at low concentrations[266].

The noteworthy effect of (poly)phenols and their metabolites in cellular signalling pathways at concentrations as low as 1 μmol has been reported already[318], reinforcing the idea of indirect mechanisms that could be involved in (poly)phenols neuroprotection. The activation of hormetic responses and effects on peripheral systems of the body, that culminate in changes in the CNS function are the proposed indirect mechanisms of action of (poly)phenols and degradation metabolites. Hormesis describes a process in which exposure to a low dose of an agent, that is toxic at higher doses, induces a beneficial effect on the cell or organism [318]. Direct evidence for hormesis induction *in vivo* has already been reported in mammals: pre-treatment of mice with epicatechin significantly reduced the negative impact of stroke induction in wild-type but not nuclear factor erythroid 2 (Nrf2) knock-out animals, as the transcription factor Nrf2 is one of the key regulators responsible for the induction of antioxidant and cell protective genes[319]. Additionally, the regulation of brain integrity and function must not be seen as an isolated process, but instead as strongly dependent on feedback information (in the form of hormones, nutrients, metabolites and, of course, sensory neuron signalling of the body periphery).

Furthermore, evidences of the metabolism of (poly)phenols at the BBB level has already been reported with the detection of the compounds conjugated with glucuronic acid[282]. The hypothesis that (poly)phenol metabolites resulting from biotransformation in endothelial cells, are the key actors in neuroprotection, arises as a novel creed in a nutritional/nutraceutical perspective. The physiological role of conjugated-flavonoids is discussible, but there is the possibility of their presence in the extracellular fluid in the CNS, and exerting biological effects. In fact, some reports have suggested that flavonoid metabolites possess more biological activity than the intact form[153, 320]. Noteworthy, biotransformation processes may constitute an essential step to convert (poly)phenols

into more water-soluble metabolites, and subsequently facilitate its elimination from the body or, oppositely, allow an easier xenobiotic transport and delivery around the body.

In a pioneer pharmacokinetics study, 23 (poly)phenols microbial metabolites were administered intravenously to rats, to reliably reproduce a physiological post absorption situation[321]. Remarkably, the brain was found to be a specific target organ for 10 of the 23 (poly)phenol metabolites injected, which significantly increased in the treated animals and most compounds were excreted into the urine[321].

In that sense, it is imperative to adjust the study of (poly)phenols beyond the reductionist dogma of (poly)phenol-effect: research should focus not only in the study of (poly)phenols but also the metabolites arising from digestion and metabolism. Dietary and colonic metabolites derived from (poly)phenols present in foods and drinks can reach considerable concentrations in circulation, usually higher than their parent compounds, and such physiologically-relevant concentrations must be studied, taking into account the multiplicity of interactions inside the human body as an overall beneficial outcome to the CNS.

5. THESIS RATIONALE

Taking into account all the evidences and all the gaps in knowledge highlighted in this introduction, the disclosure of important mechanisms behind (poly)phenols metabolites BBB putative transport and ultimate neuroprotection is imperative.

Since several epidemiological studies indicate that regular intake of fruits and vegetables can beneficially prevent brain aging-associated cognitive dysfunction, berries emerged as a rich source of phytochemicals that offer antioxidant, anti-inflammatory and direct effects in the brain, with evidences of mild cognitive impairment improvement[59]. Previous studies from our lab confirmed the brain health potential of blackberry-driven (poly)phenols (BDP) in a simple neurodegeneration cell model[220, 221]. BDP can be obtained by an *in vitro* digestion procedure, a reproducible method that has been validated against *in vivo* studies and comprises major chemical and biochemical alterations that occur in the upper gastrointestinal tract[322]. Therefore, BDP will mimic the

metabolites available in the bloodstream, which can reach the luminal surface of the BBB and therefore could cross it or interact with cell surface receptors.

Moreover, and in line with this, in a human intervention study conducted in our lab, the main bioavailable metabolites arising from the ingestion of (poly)phenols present in berries fruits were identified[323, 324]. These circulating metabolites, mainly simple phenolic sulfates, emerged as obvious targets for the assessment of BBB permeability and neuroprotection, in parallel with BDP.

Once the BBB is a dynamic and complex interface between blood and the CNS, strictly controlling exchanges, playing a key role in homeostasis and providing protection against toxic compounds[260], BDP and berry-derived bioavailable (poly)phenol metabolites transport across the BBB may be limited. The proper understanding of their BBB putative transport would clarify in deeper extent their ultimate neuroprotective potential and help to identify the key (poly)phenol metabolites responsible for such benefits. Endothelial cells of BBB, human brain microvascular endothelial cells (HBMEC), have a unique pattern of receptors and transport systems that facilitate the uptake of nutrients and hormones, in addition to active pumps that help to regulate the ions, metabolites and xenobiotics levels in the brain[257]. Due to their properties and location, endothelial cells are key players in BBB, considered as the anatomic basis of it and are widely used as simplified *in vitro* model for BBB transport assessment.

Besides the understanding of BBB transport of BDP and of berry-derived bioavailable (poly)phenol metabolites, their potential against several hallmarks of NDs must be clarified. The prevention and treatment of NDs, characterized by a mechanistic complexity, will needs novel therapeutic strategies targeted for multiple genes and proteins, such as (poly)phenol metabolites can potentially do. As more physiologically-relevant molecules, with potential to reach brain parenchyma, their role against oxidative stress, glutamate excitotoxicity and neuroinfammation, using superior and more complex cell models to modulate neurodegeneration, should be pursued. If such potential emerges, their potential in a specific NDs should be further analysed.

Among the several life-threatening and debilitating NDs, PD arises as a chronic, progressive movement disorder that primarily affects dopaminergic neurons. The understanding of PD pathogenesis, as well as the development and validation of novel

therapies, have traditionally relied on genetic modified mouse models and primary neuronal cell cultures derived from rodents, which often diverge considerably from that of human phenotype[325] and poorly mimic human disease progression *in vivo*. Human pluripotent stem cells, with their ability to differentiate in all human cellular phenotypes, hold great potential for treating PD through cell replacement therapies. Moreover, these cells are highly relevant to be used as a model to study the disease pathology.. In this sense, (poly)phenol metabolites potential towards PD will be evaluated in a more physiologically-relevant human cell model of PD. This improved model is based on 3D dopaminergic differentiation of Lund human mesencephalic neural progenitor cells (LUHMES) and treatment with the dopaminergic neurotoxicant 1-methyl-4-phenylpyridinium (MPP^+)[326, 327]. LUHMES-derived dopaminergic neurons have been extensively characterized and are morphologically and functionally similar to mature human dopaminergic neurons[328, 329].

6. AIM OF THE THESIS

This work focused on the study of blackberry and berry-derived bioavailable (poly)phenol metabolites with neuroprotective potential, especially towards PD, exploring their blood-brain barrier transport and mechanism of action. To achieve this major goal, intermediate aims were determined:

- Assess BDP transport across the BBB; evaluate BDP cytoprotective and neuroprotective potential in relevant cell models comprising different hallmarks of NDs; as well as understand gene expression alterations caused by BDP pre-incubation in concentrations close to the physiologically-described range.

- Evaluate berry-derived bioavailable (poly)phenol metabolites BBB transport in circulating concentrations; disclose their putative further metabolism by BBB endothelial cells; unveil bioavailable (poly)phenol metabolites cytoprotective and neuroprotective potential in relevant cell models with increased complexity comprising different hallmarks of NDs.

- Study the neuroprotective potential of berry-derived bioavailable (poly)phenol metabolites against PD using an improved three-dimensional cell model of the disease; disclose the molecular mechanism behind bioavailable (poly)phenols neuroprotection in PD.

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Chapter 2

In vitro digested blackberry polyphenols cross the blood-brain barrier, are neuroprotective and modulate canonical pathways

This chapter is based on the following manuscript:

Blood-brain barrier transport and neuroprotective potential of blackberry-digested polyphenols – an *in vitro* study

Figueira I, Tavares L, Jardim C, Costa I, Terrasso AP, Almeida AF, Govers C, Mês JJ, Gardner R, Becker JD, McDougall GJ, Stewart D, Filipe A, Kim KS, Brites D, Brito C, Brito MA, Santos CN. *European Journal of Nutrittion* (accepted)

This chapter contains data in which the author of this dissertation executed the majority of the experiments. Fig. 3A was performed by I. Costa and Fig. 3B was performed by A. P. Terrasso.

Table of Contents

Abstract

Epidemiological and intervention studies have attempted to link the health effects of a diet rich in fruits and vegetables with the consumption of polyphenols and their impact in neurodegenerative diseases. Studies have shown that polyphenols can cross the intestinal barrier and reach concentrations in the bloodstream able to exert effects *in vivo*. However, the effective uptake of polyphenols in the brain is still regarded with some reservations. Here we describe a combination of approaches to examine the putative transport of blackberry-digested polyphenols (BDP) across the blood-brain barrier (BBB) and ultimate evaluation of their neuroprotective effects.

BDP was obtained by in vitro digestion of blackberry extract and BDP major aglycones (hBDP) were obtained by enzymatic hydrolysis. Chemical characterization and BBB transport of extracts were evaluated by LC-MS^n. BBB transport and cytoprotection of both extracts was assessed in HBMEC monolayers. Neuroprotective potential of BDP was assessed in NT2-derived 3D co-cultures of neurons and astrocytes and in primary mouse cerebellar granule cells. BDP-modulated genes were evaluated by microarray analysis.

Components from BDP and hBDP were shown to be transported across the BBB. Physiologically-relevant concentrations of both extracts were cytoprotective at endothelial level and BDP was neuroprotective in advanced 3D cell models. The major canonical pathways involved in the neuroprotective effect of BDP were unveiled, including mTOR signaling and the unfolded protein response pathway. Genes like ASNS and ATF5 emerged as novel BDP modulated targets.

BBB transport of BDP and hBDP components reinforces the health benefits of a diet rich in polyphenols in neurodegenerative disorders. Our results suggest some novel pathways and genes that may be involved in the neuroprotective mechanism of the BDP polyphenol components.

Introduction

The increase in life expectancy in developed countries has increased the incidence of age-related diseases, especially neurodegenerative diseases. Those diseases are becoming a major problem in terms of health and wellbeing with a substantial impact on welfare care costs. In the past few years, studies have shown that dietary supplementation with fruits and vegetables, in particular berries, have effects on brain health[1]. A growing body of preclinical and clinical research identifying neurological benefits associated with the consumption of berry fruits, rich sources of (poly)phenols, have shown their potential in preventing age-related neurodegeneration, by changing cognitive and motor functions[2]. Taking advantage of cell and animal models, berry fruits have been shown to mediate signaling pathways involved in inflammation and cell survival[3], in addition to neuroplasticity and neurotransmission enhancement, leading to attenuation of age- and pathology-related deficits in behavior[1, 4].

A large number of signaling pathways and protein kinase cascades (including protein kinase C, Nrf2/ARE antioxidant pathway, pro-survival MEK/ERK and PI3K/AKT pathways), have been reported to be affected by phenolic compounds[5]. However, many reports are based on *in vitro* cell assays that study single compounds at relatively high doses, mainly as they occur in food[6]. Although these studies are important to unravel the potential mechanisms involved, such components are unlikely to reach these levels *in vivo*. (Poly)phenols are normally ingested as mixtures of different compounds immersed in a complex food matrix that undergoes a digestion process, and (poly)phenols undergo additional alterations and overcome various barriers before reaching the target organ. Following ingestion, absorption from the digestive tract often requires the hydrolysis of glycoside conjugates by small intestine enzymes (*e.g*. lactase phlorizin hydrolase or cytosolic β-glucosidase) giving rise to the corresponding aglycones[7, 8]. These aglycones can then be further metabolized by phase II enzymes, giving rise to methylated, sulfated and glucuronidated compounds [9]. On the other hand, (poly)phenols that are not absorbed in the small intestine can reach the colon and then be biotransformed by the colonic microbiota into simpler metabolites that are absorbed and further metabolized [10].

Importantly, to exert their beneficial effects in target tissues and cells, (poly)phenols and their metabolites must be both bioaccessible and bioavailable. Bioaccessible can be defined as being released and surviving gastrointestinal digestion and therefore (bio)accessible in the gut lumen. Bioavailable can be defined as being taken up into the serum and into systemic circulation. It is imperative to understand the pharmacodynamics and pharmacokinetics of dietary (poly)phenols, like any other brain-targeted drug, and determine their ability to cross the blood-brain barrier (BBB) and their ultimate protective role inside the brain. Recent studies have started to address this issue, evaluating the effective targeting of isolated parent (poly)phenols and (poly)phenol metabolites to the brain[11-15]. In this study, the blackberry extracts were subjected to an *in vitro* digestion procedure that simulates the major physiochemical effects of gastrointestinal digestion[16]. The resulting *in vitro* digested molecules represent the bioaccessible components that could putatively reach the serum by passive diffusion.

Previous work confirmed that similar bioaccessible components from blackberry promote protective actions in neuronal cells[17]. This protection was not related with enhanced intracellular antioxidant capacity nor glutathione redox status, but another mechanism was proposed[17], *i.e.* that these components caused a pre-conditioning or hormetic situation, inducing mild stress, which ultimately triggered downstream mechanisms, priming the cells against future stresses[17]. The aim of this work is to assess if these components can be transported across the BBB endothelium and also to test if they exert further neuroprotective effects using more physiologically-relevant cell models with different degrees of complexity. A transcriptomic approach was also exploited to identify major genes and networks, which were modulated by blackberry-digested (poly)phenols (BDP) and that may be responsible for any neuroprotective effects.

Results

Composition of BDP and hBDP metabolites

Blackberry-digested (poly)phenols (BDP) were obtained by in vitro digestion of blackberry extract. BDP mimics the composition of components bioaccessible in the gastro-intestinal tract, available for uptake into the bloodstream through passive transport. Enzymatic hydrolysis of BDP was also performed to obtain a more physiologically-relevant bioaccessible fraction, hBDP, which mimics the deglycosylation reactions that occur in the intestine.

Both BDP and hBDP were chemically characterized by LC-MS^n (**Fig. 1** and **Table 1**) and major peaks identified by comparing PDA profiles, m/z values and fragmentation pattern with literature data. LC-MS^n analysis of BDP confirmed the presence of glycosylated derivatives of quercetin, cyanidin, kaempferol and myricetin, as already reported[17] but hBDP lacked most of these derivatives, being enriched in the corresponding aglycones, and other breakdown products.

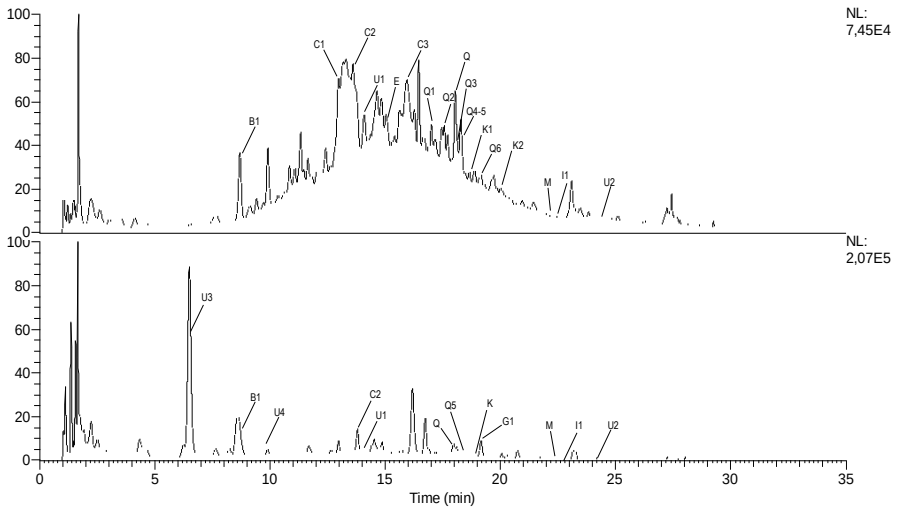


Fig. 1 PDA profile of blackberry-digested (poly)phenols (BDP – top chromatogram) and hydrolyzed BDP (hBDP – bottom chromatogram). Representative traces recorded at 280 nm are presented and the full scan deflection is shown in the upper right corner of each panel. Peaks are labelled as described in Table 1.

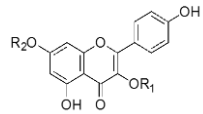
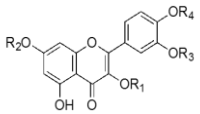
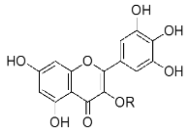
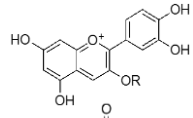
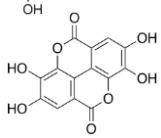
Table 1 Putative identification of major components of BDP and hBDP. Chemical profiling of BDP and hBDP was performed by LC-MS^n and major peaks identified by their PDA, *m/z* and fragmentation pattern data. Peaks named as in Fig 1. Bold = main *m/z* or MS^2 signal.

	Compound nomenclature	PDA	MS^2	m/z	RT (min)
K	Kaempferol	278	285	165	18.66
K1	Kaempferol-rutinoside	350	593	285	18.58
K2	Kaempferol-glucuronide	350	461	285	19.92
Q	Quercetin	350	301	257, **185**	18.09
Q1	Quercetin-xyloside	355	433	301	17.03
Q2	Quercetin-rutinoside	355	609	301	17.62
Q3	Quercetin-glucoside	355	463	301	18.2
Q4	Quercetin-galactoside	355	463	301	18.36
Q5	Quercetin-glucuronide	355	477	301	18.31
Q6	Quercetin-HMG-glucoside	355	**607**, 463	**463**, 301	19.17
M	Myricetin	265-360	317	317, **301**	22.19
I1	Isorhamnetin xyloside (+formate)	350	493	447, **315**	22.57
C1	Cyanidin-3-*O*-glucoside	515	449(+)	287	13.13
C2	Cyanidin-3-*O*-rutinoside	520	595(+)	287	13.57
C3	Cyanidin derivative	520	477(+)	287	16.05
E	Ellagic acid	365	301	189	15.22
B1	Dihydroxybenzoic acid	265	153	109	8.8
G1	Gallic acid derivative	275	305	169	19.16
U1	Unknown	222-276	431	385	14.15
U2	Triterpenoid derivative	ND	725	679, **517**	24.37
U3	Unknown	284	127	109	6.51
U4	Unknown	265	127	99, 83	9.91

Metabolites of both BDP and hBDP are transported across the BBB endothelium

Transport of BDP and hBDP components across confluent monolayers of human brain microvascular endothelial plated on semi-permeable membranes, a well-established in vitro BBB model[18-20] was assessed and results are indicated in **Table 2**. The kaempferol derivatives, kaempferol rutinoside and kaempferol glucuronide present in BDP were not detected in the basal compartment, suggesting that they were not transported across the monolayer. However, the aglycone kaempferol, which was not detected in the original BDP sample, was detected in basal compartment at both 2h and 24h. Moreover, in hBDP samples, which contained the aglycone kaempferol but no glycosides, the levels of kaempferol increased with time from 2h to 24h. On the other hand, all the quercetin glycosides derivatives present in BDP, except for quercetin xyloside and quercetin HMG-glucoside, were detected in the basal compartment after 2h and 24h. Moreover, the transport of quercetin-rutinoside seemed to increase with incubation time. However, the quercetin aglycone was not detected on the basal compartment when either BDP or hBDP samples were applied. Conversely, after application of hBDP, which lacked quercetin glycosides (due to enzymatic hydrolysis), both quercetin glucoside and galactoside were detected in the basal compartment. Myricetin and myricetin xyloside were present in both BDP and in hBDP. With hBDP, myricetin transport across BBB endothelium increased over the incubation time. Cyanidin derivatives were not detected in the basal compartment after 2h or after 24h of incubation. Ellagic acid transport across the BBB was also not detected. For both the digested extracts, the main transported metabolites were quercetin glucosides, kaempferol, myricetin and myricetin glucosides, and these components are therefore potential candidates to exert beneficial effects within the brain.

Table 2 Blood-brain barrier endothelial transport of major (poly)phenol metabolites identified in BDP and hBDP based on basal compartment detection. Substituents positions were inferred based on the information reported in the Human Metabolome Database (http://www.hmdb.ca/).

		Presence		Relative change* (24 *vs* . 2h)	
Compound Structure	**Compound nomenclature**	**BDP**	**hBDP**	**BDP**	**hBDP**
	Kaempferol ($R_{1,2}$=H)	n.d.	✔	0.98	**1.4**
	Kaempferol-rutinoside (R_1=rutin, R_2=H)	✔	n.d.	n.d	n.d
	Kaempferol-glucuronide (R_1=glucuronic acid, R_2=H)	✔	n.d.	n.d.	n.d.
	Quercetin (R_{1-4}=H)	✔	✔	n.d.	n.d.
	Quercetin-xyloside ($R_{2,3,4}$=H, R_1=xylose)	✔	n.d.	n.d.	n.d.
	Quercetin-rutinoside (R_1=rutin, R_{2-4}=H)	✔	n.d.	**1.4**	n.d.
	Quercetin-glucoside (R_{2-4}=H, R_1=glucose)	✔	n.d.	0.7	0.46
	Quercetin-galactoside (R_1=galactose, R_{2-4}=H)	✔	n.d.	0.87	0.57
	Quercetin-glucuronide (R_1=glucuronic acid, R_{2-4}=H)	✔	✔	0.64	0.53
	Quercetin-HMG-glucoside (R_1=HMG-glucose, R_{2-4}=H)	✔	n.d.	n.d.	n.d.
	Myricetin (R=H)	✔	✔	0.48	**1.9**
	Cyanidin-3-O-glucoside (R= glucose)	✔	n.d.	n.d.	n.d.
	Cyanidin-3-O-rutinoside (R=rutin)	✔	✔	n.d.	n.d.
	Cyanidin derivative	✔	n.d.	n.d.	n.d.
	Ellagic acid	✔	n.d.	n.d.	n.d.

* ✓- presence detected in basal compartment; n.d. – not detected; relative change ratioed for 1.0 at 2h of incubation.

BDP and hBDP protect human brain microvascular endothelial cells against oxidative insult

To evaluate the protective potential of BDP and hBDP at the luminal face of brain capillaries of the BBB, HBMEC were exposed to an oxidative insult after incubation with either BDP or hBDP. Pre-incubation of HBMEC with physiologically-relevant concentrations of BDP[9, 13, 21, 22] for 24h protected the cells from the oxidative damage caused by hydrogen peroxide (**Fig. 2a**). Moreover, hBDP also revealed protective activity at concentrations greater than

1.25 µg GAE/mL (**Fig. 2b**). Indeed, BDP and hBDP treatments restored cell viability to levels comparable with control cells (**Fig. 2a** and **2b**).

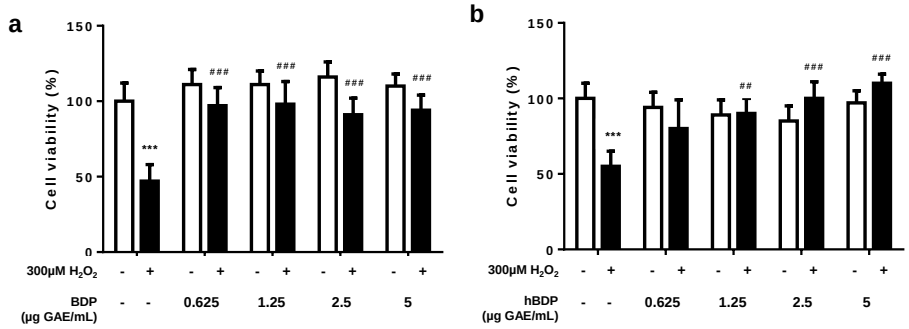


Fig. 2 Cytoprotective potential of BDP (**a**) and hBDP (**b**) in the HBMEC line. Cells were pre-incubated with different concentrations of BDP or hBDP (0.625-5 µg GAE/mL) for 24h and then submitted to oxidative stress (300µM H_2O_2). Cell viability was assessed and is presented as percentage relative to control. Statistical differences are denoted as ***p<0.001, relative to control, and ###p<0.001 and ##p<0.01 relative to H_2O_2 treatment. Data are presented as means ± SD, n=3.

BDP is neuroprotective against oxidative insult and excitotoxicity

We further explored the neuroprotective potential of BDP using a more physiologically relevant cellular model comprising of a mixture of neurons and astrocytes in a three-dimensional architecture (**Fig. 3**)[23, 24]. The cell viability of the 3D co-cultures was greatly reduced after oxidative stress induction with *t*-BHP (**Fig. 3a**). However, pre-incubation with BDP, prior to the oxidative insult, significantly protected the neuronal cells from the oxidative injury (**Fig. 3a**).

The neuroprotective effect of BDP in an excitotoxic environment was also explored, a scenario never reported before for these components. Primary cultures of mouse cerebellar granule cells pre-incubated with BDP were injured with glutamate to cause excitotoxicity-related damage (**Fig. 3b**). Of the three physiologically-relevant concentrations of BDP used, only the highest (1µg GAE/mL) significantly protected cells from the damage caused by glutamate (**Fig. 3b**).

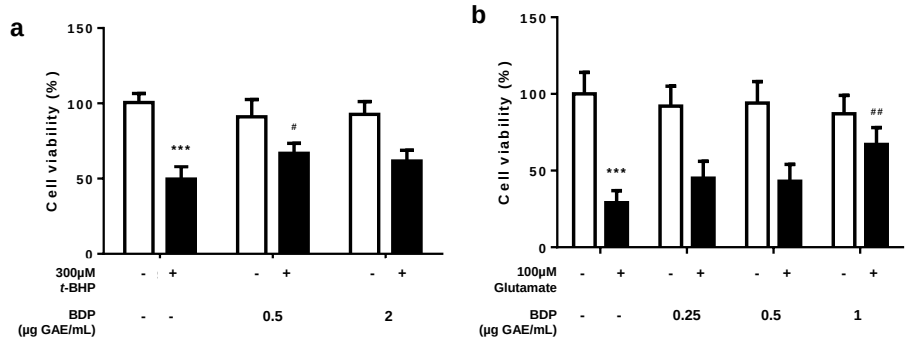


Fig. 3 Neuroprotective potential of BDP in NT2 aggregates (**a**) and in primary neuronal cultures (**b**). NT2 aggregates were pre-incubated with different concentrations of BDP (0.5-2 µg GAE/mL) for 24h and then submitted to oxidative stress (300 µM *tert*-butylhydroperoxide, *t*-BHP). Primary mouse cerebellar granule cells were pre-incubated with different concentrations of BDP (0.25-1 µg GAE/mL) for 24h and then submitted to an excitotoxic stimulus (100 µM glutamate). Cell viability was assessed and is presented as percentage relative to control. Statistical differences are denoted as ***p<0.001, relative to control, and ##p<0.01and #p<0.05 relative to glutamate insult. Data are presented as means ± SD, n=3.

Differentially expressed genes induced by BDP in neuronal cells

As we have already demonstrated the protective potential of BDP in SK-N-MC cells [25], we evaluated BDP-driven gene expression alterations in these cells by microarray analysis. The number of genes whose transcription was altered (p <0.05) by BDP pre-treatment was 1124 from a total of 19557 genes, 663 being significantly up-regulated and 461 down- regulated. BDP treatment induced changes in transcription with relatively low fold change (ranging from -2.554 to 2.549).

A set of genes with the highest significance levels and expression ratios was selected for validation by RT-qPCR (**Table 3**). *HPRT1*, *GAPDH* and *B2M* were used as reference housekeeping genes for fold change normalization. Our validation results were in accordance with the microarray data and gave alterations in the same order of magnitude, with calculated *p*-values that validate our results (**Table 3**).

Table 3 Comparison of gene expression ratios in response to BDP treatment as determined by microarrays analysis and RT-qPCR.

Gene symbol	Microarray		RT-qPCR	
	FC	*p*-value	FC	*p*-value
ESRP1	2.36	0.000524	2.882	0.00398
PPIL6	2.35	2.38E-06	1.945	0.00098
ALDH1L2	2.3	3.12E-07	1.845	0.02143
ASNS	2.28	7.78E-08	3.614	0.07206
ASS1	2.23	3.06E-05	2.877	<0.0001
ATF5	2.21	6.82E-05	1.476	0.05147
PAG1	2.14	2.35E-06	1.842	0.00018
NQO1	2.12	5.84E-06	2.659	0.02104
SERPINF1	2.08	9.19E-05	2.495	0.00050
SNORD56B	-2.07	0.014491	-1.669	0.00751

Functional enrichment analysis

Functional enrichment analysis was performed on microarray data using Ingenuity® Pathway Analysis (IPA) software. Enrichment analysis on Canonical Pathways of IPA identified the significant over-represented pathways across all differentially expressed genes (**Fig. 4**). In this analysis, *Cell cycle control of chromosomal replication* was identified as the top canonical pathway, with the highest –log (*p-value*). This contained mainly downregulated genes, such as *CDC6* (cell division cycle 6), *ORC1* (origin recognition complex subunit 1), *ORC6* (origin recognition complex subunit 6), *MCM* (minichromosome maintenance complex), genes involved in pre-replication complex assembly, as well as *CDC45* (cell division cycle 45), *RPA* (replication proteins A), required at G1-S phase transition.

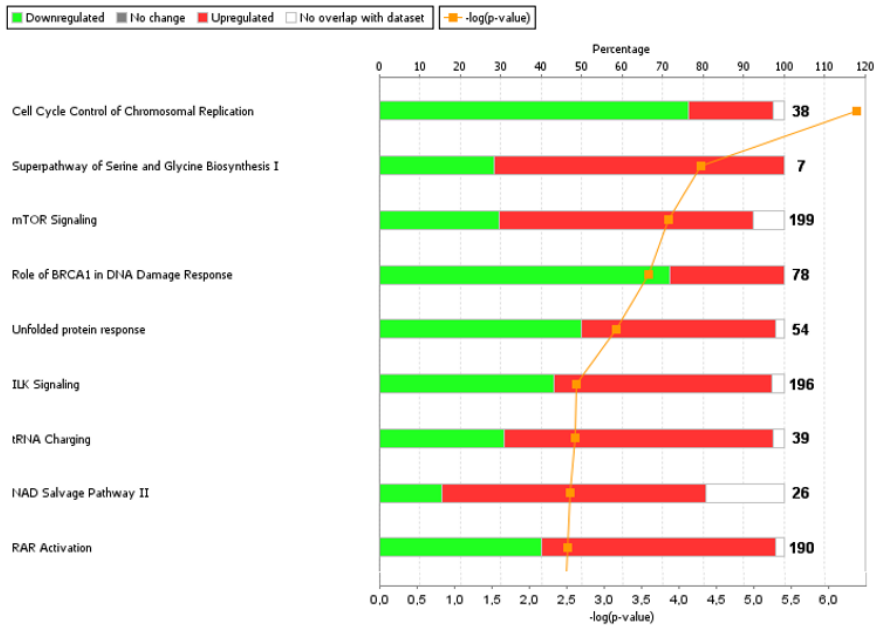


Fig. 4 Canonical pathways significantly modulated by BDP. Cells were treated with BDP for 24 h. Fisher's exact test was used to calculate a *p*-value determining the probability that the association between the genes in the data set and the canonical pathway is explained by chance. Stacked histograms display % of up- (*red*) and down (*green*) -regulated genes in a given pathway. Values at the right side indicate the number of genes involved in each particular pathway.

mTOR signaling was also identified as one of the affected canonical pathways, with the highest number of genes involved, and the related *unfolded protein response* pathway was also selected. Examining in detail the *mTOR* pathway, there were major effects arising from BDP treatment related to the upregulation of eukaryotic translation initiation factor 4EBP, together with PDL, RAS, eIF3, AMPK and VEGF, and downregulation of the mammalian target of rapamycin (mTOR), phosphatidylinositol-3-kinases (PI3K), IRS1 and eIF4G (**Fig. 5**). Therefore, this analysis predicts final outcomes resulting from BDP treatment, such as induction of protein translation, autophagy regulation, and regulation of VEGF signaling and of PI3K/AKT signaling (**Fig. 5**).

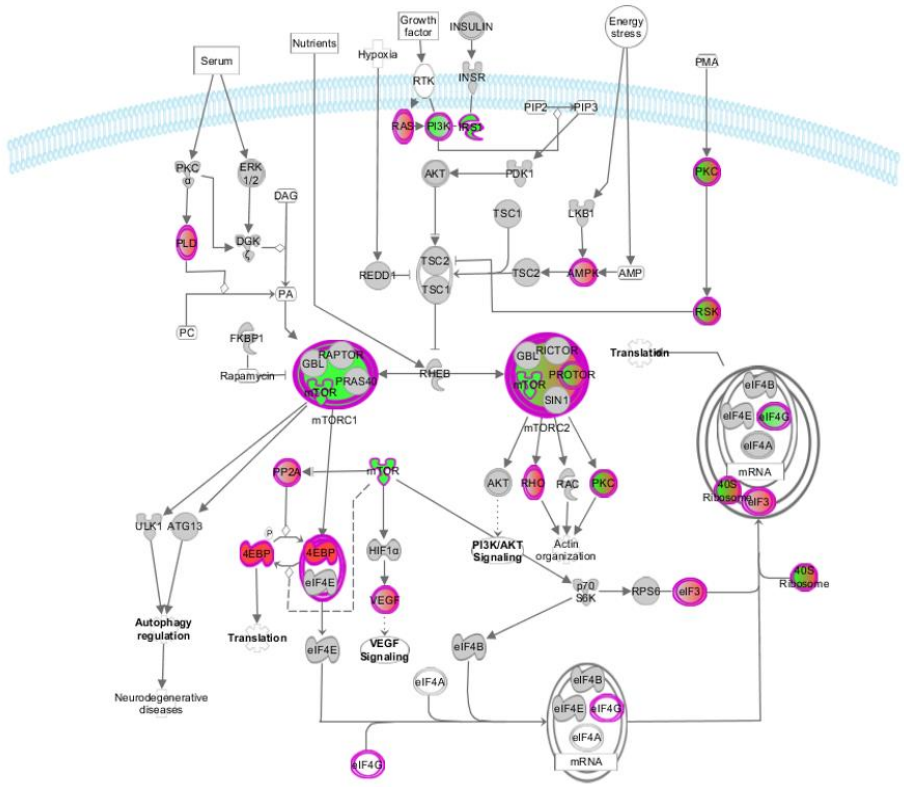


Fig. 5 Canonical pathways representing *mTOR signaling* obtained with Ingenuity® Pathway Analysis (IPA) from the analysis of dataset modulated by BDP. Genes whose expression was significantly modulated by BDP are depicted (purple) together with others involved in the cascade (but not significantly modulated, depicted in grey). Complexes or groups of genes are surrounded by a double line. Up-regulated genes are depicted in red and down-regulated genes are depicted in green. Note: some complexes/groups depicted have both up- and down-regulated genes.

The *Unfolded protein response* pathway (**Fig. 6**), related to autophagy regulation mechanisms, was another major canonical pathway over-represented after BDP treatment. BDP modulation of genes in unfolded protein response signaling pathway indicates an attenuation of translation, modulation of lipid accumulation and reduction of apoptosis (**Fig. 6**).

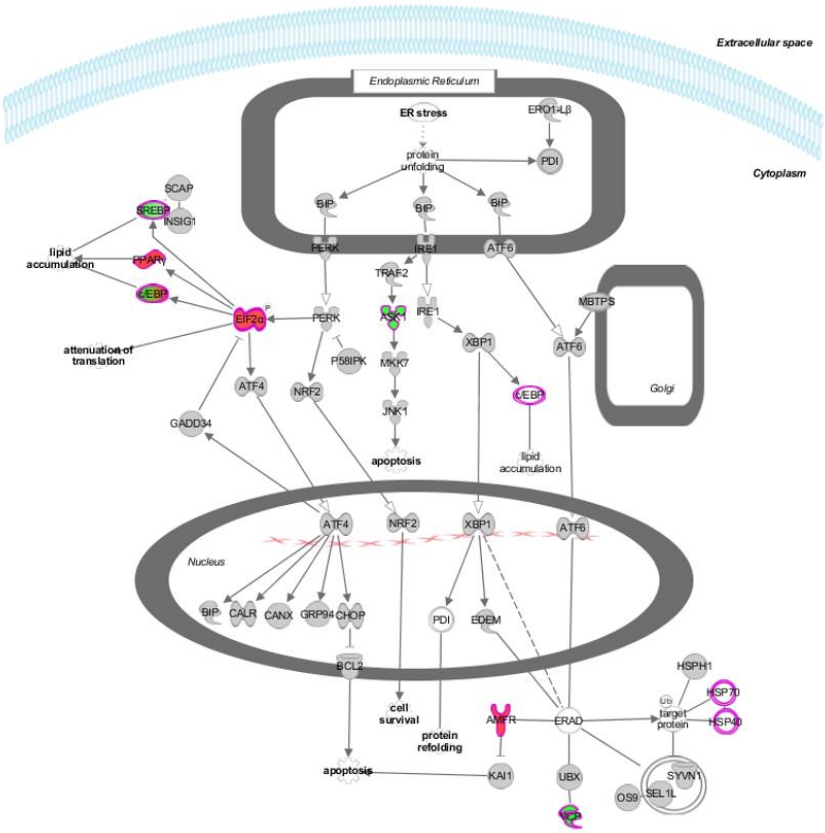


Fig. 6 Canonical pathway representing *unfolded protein response* obtained with Ingenuity® Pathway Analysis (IPA) from the analysis of dataset modulated by BDP. Genes whose expression was significantly modulated by BDP are depicted (purple) together with the others involved in the cascade (but not significantly modulated, depicted in grey Complexes/groups of genes are surrounded by double lines. Up-regulated genes are depicted in red and down-regulated genes are depicted in green. Note: some complex/group depicted have both up- and down-regulated genes.

The Upstream Regulator Analysis tool infers the modulation of enzymes, kinases and transcription regulators that could explain the observed gene expression changes (**Table 4**). The cascade of events that lead to these gene expression alterations presumes the early involvement of these regulators. TP53 and ATF4 were two transcription regulators with the highest z-score and with significant *p*-value of overlap suggesting activation (**Table 4**). Moreover, this tool also predicted the inhibition of upstream regulators, among which E2F1 and CCND1 presented the lowest z-score and also significant *p*-value of overlap (**Table 4**). Among the downstream target genes regulated by TP53, the major significantly upregulated in our dataset were asparagine synthetase (glutamine-hydrolyzing, *ASNS*), annexin A4 (*ANXA4*), protein tyrosine

phosphatase type IVA - member 3 (*PTP4A3*), and ribosomal protein S6 kinase A2 (*RPS6KA2*), and the major downregulated ones were protein kinase D1 (*PRKD1*) and A-kinase anchoring protein 12 (*AKAP12*).

Table 4 Upstream regulator analysis obtained from the gene expression dataset from cells incubated with BDP, using IPA software. Predicted activation state correlates with calculated z-score, which if it is above or equal to 2 corresponds to an activation, and if it is below or equal to -2 corresponds to an inhibition.

Upstream regulator	Molecule Type	Predicted activation state	Z-score	Number of target genes in dataset
TP53 (tumor protein p53)	transcription regulator	Activated	4.321	172
E2F1 (transcription factor E2F1)	transcription regulator	Inhibited	-3.645	74
CCND1 (cyclin D1)	transcription regulator	Inhibited	-4.313	52
ATF4 (activating transcription factor 4)	transcription regulator	Activated	3.822	34
CDKN1A (cyclin dependent kinase inhibitor 1A)	kinase	Activated	2.076	45
NUPR1 (nuclear protein 1)	transcription regulator	Activated	3.077	78
Vegf (vascular endothelial growth factor)	growth factor (group)	Inhibited	-2.4	74
E2f (E2F transcription factors)	transcription regulator (group)	Inhibited	-3.472	29

Genes such as *ASNS* (also under control of TP53) and *ATF5*, which were strongly upregulated by BDP, are downstream of ATF4 (Fg. 7). Moreover, ATF4 also modulates genes involved in the “unfolded protein response pathway” (*CEBPB, CEBPG, SREBF1*), in the “asparagine and serine pathway” (*ASNS, PHGDH, PSTA1, PSPH, SHMT2*), and in “tRNA charging and processing pathway” (*GARS, IARS, SARS, WARS, XPOT*) (**Fig. 7**). In addition, downstream of ATF4 is the central signaling hub *JUN* which was downregulated by BDP (**Fig. 7**).

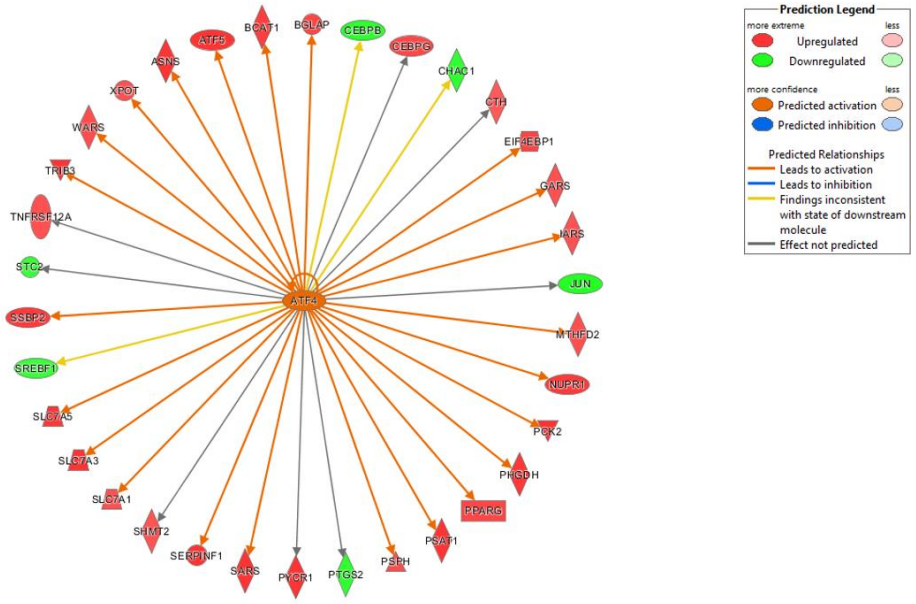


Fig. 7 Identification of ATF4 as an upstream regulator of transcriptional regulation by BDP. Bioinformatic analysis with IPA software predicted the activation of ATF4 (orange) as a regulator of genes that are upregulated (28, depicted in red) and downregulated (6, depicted in green). Arrows represent predicted relationships: orange – leads to activation; yellow – findings inconsistent with state of downstream molecule; grey – effect not predicted.

Discussion

Blackberry extract was subjected to an *in vitro* digestion procedure that mimics the physiochemical alterations that occur in the gastro-intestinal tract. Although it cannot mimic the dynamic changes occurring in the gastro-intestinal tract or active processes such as uptake[26], including gastric absorption of anthocyanins, which can influence serum levels[27], this procedure captures the major changes in (poly)phenol stability to digestion that occur *in vivo*. It is a reproducible method[16, 17] and recent *in vivo* studies on ileal fluids after raspberry intake[28] indicate that it replicates the major alterations including ellagitannin stability and anthocyanin degradation. We have already described that BDP improved neuroprotective capacity, which may arise due to the phenolic composition alteration as a consequence of digestion (*i.e.* lower levels of anthocyanins and ellagitannins but relatively enhanced levels of quercetin derivatives, with evidence of accumulation of breakdown products)[17]. However, as intestinal absorption is not taken into account in the *in vitro* digestion method used, we obtained hBDP which mimicked the enzymatic hydrolysis of glycoside conjugates that can occur in the intestine[7, 8, 21]. .

It is not yet completely clear if (poly)phenol metabolites can cross the BBB by simple diffusion or by specific carrier-mediated transport, or how structure influences brain bioavailability[29]. Previous work from our lab demonstrated that differences in endothelial transport could be related with the degree of chemical modification, where methylation combined with sulfation enhanced the transport of bioavailable (poly)phenol metabolites[30]. Moreover, the presence of efflux transporters at BBB endothelial level can also limit (poly)phenol metabolites transport from the blood to the brain[30].

The results from BBB endothelial transport of BDP suggest that the glycosides, glucuronic acid and rutinoside, may be removed from the kaempferol glycosides during transport. Indeed, animal studies with *Ginkgo biloba* extract EGb 761®, rich in quercetin and kaempferol glycosides, revealed that kaempferol reached the brain of the rats after single oral administration and C_{max} was reached 8h after ingestion[11].

The detection of quercetin glucosides (quercetin glucoside and galactoside) in the basal compartment after application of hBDP samples, which are devoid of these components, suggests that these glycosides may be added by cellular activities. Indeed, enzymes

such as UDP-glucuronosyltransferase, capable of metabolizing (poly)phenols into glucuronide derivatives, have been described in the BBB endothelium[30, 31]. In fact, enzymes primarily associated with hepatic drug metabolism have been shown to exist in the brain, albeit at relatively low specific activities[32] and may influence cerebral availability of various compounds at the blood-brain interface[31, 33].

There were clear differences in the BBB endothelial transport of kaempferol and myricetin compared to quercetin. Kaempferol, quercetin and myricetin are flavonols and differ only in the number of hydroxyl groups in the B-ring (1, 2 or 3 -OH, respectively). Intermolecular hydrogen bonds can form between the two hydroxyls at the B-ring of quercetin[34] and the same could occur between two of the three hydroxyls in the B-ring of myricetin. We can speculate that the "free" hydroxyl group in kaempferol and myricetin could influence its BBB permeation; however, other factors may contribute to explain the pattern of transport specificity noted. As these flavonol components are present in mixtures and at differing concentrations, it is possible that they, or other components (such as the cyanidins), interfere with each other's transport. It is also possible that certain components (e.g. the acylated HMG-glucoside of quercetin) are less stable in the cell media and are degraded before they can be transported. Moreover, in such mixtures, certain components (such as anthocyanins) may be more sensitive to oxidative degradation. The stability of components could be examined by monitoring recovery from media and transport of degradation products examined using radio- or isotopically labeled components. Despite the accumulating evidence for free quercetin detected in the brain after oral administration[22, 35], we propose that it may result from degradation of conjugated forms, which are transported across the BBB, and quercetin alone may not be able to be transported. In fact, in a previous study[36] we have detected the transport of quercetin glucosides but not of free quercetin. Accordingly, a very low efficiency of free quercetin transport (around 8% after 18 h incubation) was reported[37] which was attributed to: (i) a low uptake at the apical membrane, (ii) reduced transport through the basolateral membrane, (iii) a high metabolism/ degradation rate, or (iv) an increased efflux[37]. Actually, quercetin has been described both as a P-glycoprotein inhibitor and/or substrate[38], which could contribute to its low BBB transport.

The fact that no cyanidin derivatives were detected in basal compartment is in line with our previous data: for example, even for higher concentrations, only 1% of cyanidin-3-*O*-glucoside crossed a confluent monolayer of HBMEC[36]. Several studies have reported the capacity of anthocyanin derivatives to reach the brain, and their pharmacokinetics is very rapid, being detected in the brain after 10 minutes from their introduction into the stomach[39]. Moreover, the biotransformation of cyanidin-3-*O*-glucoside is very fast and substantial, with the appearance of methylated derivatives such as peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside, both in plasma and in the brain [15]. We can speculate that such metabolism may occur at the BBB level, but we cannot confirm this from our LC-MS^n data.

Enzymatic hydrolysis of BDP may lead to a more bioavailable fraction in terms of intestinal absorption but, once in circulation, the aglycones present will be further metabolized and the end-products that ultimately can reach the luminal surface of endothelial cells of brain capillaries of the BBB may be methylated, sulfated and/or glucuronidated[9, 40]. Our data demonstrates that (poly)phenol components arising from blackberry extract after *in vitro* digestion (BDP) and subsequently enzymatically hydrolyzed (hBDP) are able to be transported across the BBB endothelium and in some cases even further metabolized.

Endothelial cells of the brain microvasculature are the "front line" in the protection the of brain cells from external agents which may lead to increased as oxidative stress, inflammation, vascular reactivity and excitotoxicity[41, 42]. Although hBDP had some protective potential at the BBB interface, BDP was more effective at lower levels, which were more comparable to those that may reach the brain [9, 13, 22], and therefore BDP was explored in greater depth. We have already evaluated the neuroprotective potential of BDP in a simplified neurodegeneration cell model[17, 25] so we extended our studies to a more physiologically relevant cell model, *i.e.* a complex cell system comprising a mixture of neurons and astrocytes with a three-dimensional architecture[23, 24]. The 3D aggregates are mainly composed of astrocytes (~77% of the cells)[23] and glial cells (astrocytes and microglia), which are responsible for the repair process of the brain in the case of injury[43], being stronger and less susceptible to damage than neurons. In our previous work, we observed that exposure to *t*-BHP caused greater damage to neurons than

astrocytes within the aggregates[39]. We argued that protection conferred by bioavailable concentrations of (poly)phenol metabolites in this system could be related to improving the functionality of the astrocytic population and not directly in the reduction of the deleterious effects caused by the oxidative injury[30].

BDP was also neuroprotective in an excitotoxicity scenario. Previous studies have reported neuroprotective effects of blueberry fruit extracts towards glutamate toxicity, but using whole fruit extracts and in concentrations higher than what might be achieved *in vivo*[44]. Another frequent approach is to test pure compounds, which is interesting for elucidating the chemical basis related with different neuroprotective effects of the compounds, but is not nutritionally translatable. To our knowledge, this is the first report of protection against excitotoxicity by bioaccessible (poly)phenol components.

Most of the *in vitro* data on the effects of (poly)phenols on molecular pathways and protein kinase cascades involved in neuroprotection have been gathered using pure (poly)phenols, in isolation and often at non-physiologically-relevant concentrations. Gene expression analysis was performed in SK-N-MC cells, where BDP has already proven to be protective[25]. BDP led to modest alterations (~ 2-fold) in gene expression, which is consistent with previously reported actions of dietary-derived compounds: which although usually complex, are characterized by the induction of a broad array of genes with modest changes in their intensity[45]. Furthermore, modest alterations may be expected as low, physiological but cytoprotective concentrations of BDP (0.5 µg GAE/mL) were used[17]. Among such genes was *NQO1,* a member of the NAD(P)H dehydrogenase (quinone) family, described to be involved in oxidative stress and antioxidant response pathways, preventing the one electron reduction of quinones that results in the production of radical species[46]. Moreover, the ubiquitin-independent p53 degradation pathway is regulated by NQO1 by stabilizing p53, protecting it from degradation[47]. In fact, tumor suppressor p53 is responsible for conserving the stability of the genome, preventing the rise of mutations in the DNA[48]. We suspect that *NQO1* upregulation can modulate p53 signaling, with ultimate benefits to cell survival. Another upregulated gene was *SERPINF1*, coding for pigment epithelium-derived factor (PEDF, also known as serpin F1), described to be anti-angiogenic, anti-tumorigenic and neurotrophic. PEDF has been shown to be neuroprotective against acute glutamate

toxicity[49], and high glucose or H_2O_2[50]. PEDF can also induce p53 through PPARγ expression, stimulating several other signaling cascades like Ras and NF-κB pathways[51]. The significant upregulation of such genes may, at least in part, justify the protective effect conferred by BDP (poly)phenols.

Functional enrichment analysis identified the major canonical pathways predicted to be modulated by BDP, such as *mTOR signaling* and *Unfolded Protein Response*. Several reports demonstrate positive effects of dietary (poly)phenols in mTOR signaling and induction of protein translation, autophagy regulation, and regulation of VEGF signaling and of PI3K/AKT signaling[52, 53]. For instance, fisetin significantly increased p-PI3K, p-Akt and p-GSK3β expression in $A\beta_{1-42}$-treated mice, accompanied by a marked reversion of synaptic dysfunction, with improvement of mouse memory[54]. A mixture of grape seed extract, Concord grape juice and resveratrol significantly improved sleep deprivation-induced cognitive impairment, in which brain-available quercetin-3-*O*-glucuronide activated CREB signaling and malvidin-3-*O*-glucoside activated mTOR signaling[55]. Our results confirm that blackberry (poly)phenols resulting from *in vitro* digestion affect these same signaling pathways with putative neuroprotective outcomes.

The upregulation of the regulatory subunit of eukaryotic initiation factor 2 (eIF2α) in unfolded protein response pathway is related with endoplasmic reticulum (ER) stress, normally through increases in its phosphorylation by PERK[56]. This regulation, mediated by BDP, suggests that translation might be slowed since initiation may be blocked. On the other hand, modulation of eIF2α has already been described to be linked to key regulators of lipid accumulation (PPARγ, C/EBP, SREBP)[56]. As lipid peroxidation is associated with brain injury, BDP may modulate lipid accumulation and then confer an adaptive advantage for further cellular stress.

Apoptosis is also an important mechanism suggested to be affected by BDP. For instance, *ASK1* codes for mitogen-activated protein kinase 5 (MAP3K5), a member of MAP kinase family, responsible for the activation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases under stress (*e.g.* oxidative stress, ER stress or calcium influx)[57]. The downregulation of *ASK1* may reduce its activity, which may lead to a decrease in apoptosis. In this case, BDP may be activating through

cytoprotective/neuroprotective mechanisms that help the cell to cope with the effects of an oxidative agent.

Another upregulated gene was chaperone Hsp40 (DnaJ), which acts through ATPase activity stimulation of Hsp70 activity, and also associates with unfolded polypeptide chains to prevent their aggregation [58]. Indeed, we also observed downregulation of Hsp70 mRNA levels. Our results suggest an Hsp40-mediated activation of the regulatory mechanism of Hsp70 activity, which is independent of Hsp70 cellular levels. This regulation could comprise an important mechanism of protection by priming the cells to cope with a later stress. Acquisition of stress tolerance by Hsps regulatory mechanisms was already described in mammalian cells pre-exposed to thermal stress[59] and by resveratrol[60]. To our knowledge, this the first study where bioaccessible (poly)phenols from blackberry fruits are described to modulate key players in the unfolded protein response.

The major upstream regulator predicted to be activated by BDP was TP53. The p53 and p53-signaling pathway has already been involved as a functional mechanism for protection using non-nutritional approaches with pure (poly)phenols[61, 62]. However it is not clear what (poly)phenols may target in the p53 regulatory pathway and how this can affect downstream metabolism. Our analysis reveals potential novel targets that were modulated by bioaccessible (poly)phenols at physiologically-relevant concentrations, including *ASNS* and *ANX4*. The only previous reports describing enhanced expression of these genes referred to very different conditions: *e.g.* effects of lignin on *ASNS* in macrophages[63] and low, non-toxic concentrations of ethanol for *ANX4* in rat glioma C6 cells and human adenocarcinoma A549 cells[64].

Activating transcription factor 4 (ATF4), involved in ER stress response, was also predicted to be activated by BDP. ATF4 plays a crucial role in stress adaptation and its major function is to mediate the induction of the integrated stress response[65, 66]. Several ATF4 target genes, such as *CHOP*, are themselves transcription factors that regulate and amplify the signal initiated by the original stress. Downstream of *ATF4* is *ATF5*, a similar but less studied ATF4 homolog. ATF4 has established roles in cell susceptibility to ER stress through the regulation of CHOP, whereas ATF5 has been found to have anti-apoptotic roles in several tissues[67, 68]. Two direct transcriptional targets of ATF5

have been identified, the anti-apoptotic factor B-cell chronic lymphocytic leukemia/lymphoma 2 (BCL-2) and mTOR[69, 70]. The dual role of ATF5 in cell survival and stress response appears as new and promising target for the study of (poly)phenol-derived metabolites. To date, there are no studies reporting the modulatory effects of (poly)phenols or (poly)phenol-derived metabolites on ATF5.

The modulatory effect of BDP on tRNA charging and processing at the level of gene expression is a novel layer of regulation for (poly)phenol components. Direct physical interactions of pure (poly)phenols with tRNA have already been described, with evidence that low flavonoid concentration induced helical stabilization[71, 72]. Jointly, these effects, when mediated by (poly)phenol components, may impact central and fine-tuned mechanisms of the cell. It was demonstrated that EGCG significantly inhibited phosphorylation of the MAPK p38 and JNK, and NF-κB and AP-1 transcriptional activities, whilst causing anti-inflammatory and anti-oxidant effects in human corneal epithelial cells[73]. Moreover, a (poly)phenol-rich peanut extract reduced extracellular TNF-α release by inhibiting c-Jun transcription factor activity[74]. BDP-predicted upregulation of ATF4, at physiologically-relevant concentrations, may be one of the most important regulatory pathways responsible for BDP neuroprotective effects against stress. Under the umbrella of ATF4, our results pinpoint some pathways already associated with (poly)phenols but also indicate novel targets for (poly)phenols, such as ATF5 or regulation of tRNA charging and processing.

Conclusion

Blackberry (poly)phenol components, resulting from a validated *in vitro* digestion protocol, are transported across the blood-brain barrier endothelium and therefore may exert neuroprotective action in situ. Neuroprotective activities were observed in more-physiological and biological relevant complex cellular models such as mixed 3D cellular culture systems. Our results support the hypothesis that exposure to chronic low levels of bioaccessible and nutritionally relevant metabolites may significantly influence key cellular mechanisms in neuronal cells in a preventive context. From gene expression analysis, we conclude that the blackberry (poly)phenol components modulated genes in canonical signaling pathways, as well as novel targets. Some of the molecular targets

identified, involved in cell cycle control, mTOR signaling and unfolded protein response, have already been described to be modulated by dietary (poly)phenols. However, to our knowledge, this is the first time that such alterations in gene regulation by bioaccessible (poly)phenol components have been described. Additionally, genes modulated by these components may indicate novel mechanistic targets for neuroprotection.

Overall, our results illustrate how bioaccessible (poly)phenol components, at physiological concentrations, may access brain cells and be protective. By revealing significant gene modulatory effects of diet-derived compounds, we enlarge our understanding of how the food we eat impacts gene expression and ultimately prevent neuronal dysfunction.

Material and Methods

Preparation of blackberry-digested (poly)phenols

Blackberry fruits (*Rubus L*. subgenus *Rubus* Watson) were grown at Fataca experimental field station (Odemira, Portugal). The frozen ripe berries were freeze-dried, ground without separation of seeds in an IKA M20 mill to pass a 0.5 mm sieve and stored at -80 ºC prior to extraction. Fruit extracts were prepared using a hydroethanolic solution (ethanol 50 %, v/v)[75]. *In vitro* digested extract [named blackberry-digested (poly)phenols (BDP)] was obtained using the previously described method in which the final digestate is obtained following passive diffusion through a dialysis membrane[3].

Preparation of hydrolyzed blackberry-digested (poly)phenols

Enzymatic hydrolysis of BDP was performed as described previously[21] with some modifications. BDP extract was acidified to pH 5.0 with acetic acid and cellulase was added to a final concentration of 20 U/mL (1 U corresponds to 0.885 mg of protein and is defined as the amount required to liberate 1.0 µmol of glucose from cellulose in 1h at pH 5.0 at 37 °C). Samples [named as the hydrolyzed blackberry-digested (poly)phenols (hBDP)] were incubated for 6 h at 37 °C and then purified by solid phase extraction.

The total phenolic content of BDP and hBDP was determined by the Folin–Ciocalteu method adapted to microplate reader[76] using gallic acid as standard and expressed as mg of gallic acid equivalents per gram of dry weight (mg GAE/g dw). Both BDP and hBDP were dried by rotary evaporation, re-suspended in 5% (v/v) acetonitrile in water and analyzed by LC-MS^n as described below.

BBB Endothelial Transport Assays

A cell line derived from primary cultures of human brain microvascular endothelial cells (HBMEC) transfected with SV40 large T antigen[77] was used as a simplified *in vitro* model of BBB. The HBMEC line was cultured as previously described[78]. For transport studies, endothelial cells were cultured on semipermeable filters (polyester transwell inserts, 0.4 µm, Corning Costar Corp., USA) placed in 12-well culture plates, which separate two compartments: the upper compartment that can be considered as the “blood-side”, where BPD or hBPD were added, and the lower (basal) compartment, which is

considered the “brain side”. Cells were seeded at a density of 8×10^4 cell/insert and treated after 8 days in culture. All experiments were performed in triplicate and after monolayer formation.

Transport assays were conducted in a specific incubation medium: HBSS (Hank's Balanced Salt Solution) with calcium and magnesium (Gibco), supplemented with 0.1% FBS. Confluent monolayers of HBMEC were incubated with 5µg GAE/mL of BDP or hBDP. In order to evaluate a time-dependent transport, BDP or hBDP were added to the upper compartment and samples from upper and lower compartments were collected after 2 and 24 h. Monolayer integrity was ensured in all experiments by monitoring transendothelial electrical resistance (TEER) and paracellular permeability [78]. No alterations were observed reflecting barrier integrity throughout the experiments. In the end, cell medium from upper and lower compartments were collected and frozen at -80 °C until analysis.

Liquid Chromatography Mass Spectrometry (LC-MS^n)

Deproteinization of samples of upper and lower compartments was performed before LC-MS^n analysis. To 1 mL of cell medium it was added 139 µL of 50% formic acid, ascorbic acid (final concentration of 1 mM) and taxifolin as internal standard (final concentration of 9 µM). To precipitate proteins, 2.5 mL of acetonitrile was added dropwise and samples were vortexed before centrifugation at 3200 *g* for 15 min. The supernatant was removed and dried under centrifugal evaporation (CentriVap Vacuum Concentrator, Labconco). Samples were dissolved in 5% acetonitrile in 0.1% formic acid and immediately analyzed on a LC-Orbitrap MS. Samples were separated on an HPLC Accela 600 HPLC system (Thermo Scientific, Bremen, Germany) using a C18 Synergi Hydro RP18 column [Phenomenex, Macclesfield, UK) 4 µm particle size and dimensions 2 mm ID × 150 mm] fitted with a Security GuardTM guard [Aqua 10 µm C18 Guard Cartridge (2mm ID×4 mm; Phenomenex)]. Samples were eluted over a gradient of 98% solvent A (0.1% formic acid in ultra-pure water) to reach 5% B (0.1% formic acid in acetonitrile) at 5 min, 35% B at 25 min, increase to 100% B at 26 min, 100% B at 29 min, and back to 2% B at 30 min at a flow rate of 0.2 mL/min. LC-MS^n analysis was carried out on an LTQ OrbitrapTM XL hybrid mass spectrometer (Thermo Scientific,

Bremen, Germany). MS analysis was performed using data-dependent Nth order double play analysis comprising full scan mass range 80–2000 amu, 30 000 resolution, data-type centroid and data dependent MS/MS (60 s of exclusion duration) on the top three most intense ions detected above threshold automatically in the independent scan event. ESI settings were as follows: source voltage, 3.4 kV; capillary temperature was 275°C with a sheath gas at 40 psi and auxiliary gas at 5 psi. MS data handling software (Xcalibur QualBrowser software, Thermo Electron Corp.) was used to search for predicted metabolites by their appropriate *m/z* value. All peaks were checked for *m/z* value and fragmentation products.

Cytoprotection assays

Cytoprotection in HBMEC

For cytoprotection studies, cells were seeded at a density of 8×10^4 cell/mL in 96-well plates. Briefly, 24 h after seeding, HBMEC cells were pre-incubated for 24h with 0.625, 1.25, 2.5 and 5 µg GAE/mL of BDP or hBDP in cell medium. After pre-incubation, medium was replaced by new medium containing 300 µM H_2O_2, for 24 h[25, 79]. In the end, cell viability was assessed using the CellTiter-Blue® Cell Viability Assay (Promega), according to the manufacturer's instructions. Final cell viability was calculated as a percentage of control cells viability.

Neuroprotection in 3D co-cultures of neurons and astrocytes

NTera-2/cl.D1 (NT2) cells (ATCC), were differentiated in stirred suspension culture systems, as 3D aggregates, as previously described[23]. Stable 3D co-cultures of human neurons and astrocytes were maintained up to day 50 of culture. For neuroprotection assays, 3D co-cultures were collected (between day 38 and 50) and distributed in 96-well plates. BDP or hBDP (0.5 and 2 µg GAE/mL) were added to the cultures and after 24 h, an oxidative insult was induced by addition of 300 µM tert-buthyl hydroperoxide (*t*-BHP; Sigma-Aldrich) for 48 h as previously described[24]. Cell viability was accessed with PrestoBlue® Cell Viability Reagent (Thermo Fisher Scientific), accordingly to manufacturer's instructions. Final cell viability was calculated as a percentage of control cells viability.

Neuroprotection in cerebellar granule cells

Primary cultures of mouse cerebellar granule cells were prepared from cerebella of 7d-old BALB-C mice as previously described[80]. Three concentrations of BDP (0.25, 0.5 and 1 µg GAE/mL) were added to the cells at 7d in culture. Cells were then incubated for 24 h and morphology was evaluated. At day 8 in culture, 100 µM of glutamate (Sigma-Aldrich) was added. Cells were incubated for another 24 h, morphology was evaluated and viability determined by fluorescence microscopy with propidium iodide (PI) and Hoechst 33342 labeling, as previously described[80]. Final cell viability was calculated as a percentage of control cells viability.

Microarray analysis

Human neuroblastoma SK-N-MC cells (ECACC) were cultured as previously described[25]. SK-N-MC cells were seeded at $5x10^4$ cells/cm^2 and grown for 24 h. BDP was supplied to cells (0.5 µg GAE/mL) dissolved in medium containing 0.5% (v/v) FBS for 24 h[25]. After incubation, cells were washed with PBS, trypsinized, collected into a tube and 0.05 volumes of FBS added to inactivate trypsin. Dihexyloxacarbocyanine iodide ($DiOC_6(3)$) and PI were added to cells at a final concentration of 20 nM and 1 µg/mL, respectively, and incubated for 30 min at 37 ºC. $DiOC_6(3)$ was used to evaluate the mitochondrial transmembrane potential ($\Delta\Psi m$) and PI to determine cell viability, based on plasma membrane integrity[25]. Cells were then sorted in a FACSAria High Speed Cell Sorter (Becton Dickinson), using a 100 µm nozzle with 206.8 kPa (30 psi) sheath pressure. A 488 nm laser was used for DiOC6(3) and PI excitation and detection was performed using a 530/30 nm and a 695/40 nm HQ band pass filter, respectively. Only cells that exhibited cellular membrane integrity and high mitochondrial transmembrane potential were collected in tubes containing PBS for further analysis (**Supplementary Fig. S1**).

Total RNA extraction of sorted cells was performed using AxyPrep Multisource Total RNA Miniprep (Axygen) as previously described[81]. Total RNA was treated with Turbo™ DNase I (Ambion), accordingly to the manufacturer's instructions. RNA quantity was

assessed using a Nano-Drop® ND-1000 spectrophotometer (NanoDrop Technologies) and RNA quality using an Agilent 2100 Bioanalyser with an RNA 6000 Nano Assay (Agilent Technologies). Biological triplicates were processed for use on Affymetrix (Santa Clara, CA, USA) GeneChip HuGene 1.0 ST Arrays at the Gene Expression Unit of Instituto Gulbenkian de Ciência (Oeiras, Portugal), and, according to the manufacturer's Whole Transcript Sense Target Labeling Assay. Briefly, 100 ng of total RNA containing "spiked in" Poly-A RNA controls (GeneChip Expression GeneChip Eukaryotic Poly-A RNA Control Kit; Affymetrix) was used in a reverse transcription reaction (GeneChip® WT cDNA Synthesis Kit; Affymetrix) to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in an *in vitro* transcription reaction to generate cRNA (GeneChip® WT cDNA Amplification Kit; Affymetrix). Fifteen µg of this cRNA was used for a second cycle of first-strand cDNA synthesis (GeneChip® WT cDNA Synthesis Kit; Affymetrix). From the single stranded cDNA, 5.5 µg was fragmented and end-labeled (GeneChip® WT Terminal Labeling Kit; Affymetrix). Size distribution of the fragmented and end-labeled cDNA, respectively, was assessed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay. Five µg of end-labeled, fragmented cDNA was used in a 100-µL hybridization cocktail containing added hybridization controls. Eighty µL of mixture was hybridized on arrays for 17 h at 45°C. Standard post hybridization wash and double-stain protocols (FS450_0007; GeneChip HWS kit, Affymetrix) were used on an Affymetrix GeneChip Fluidics Station 450. Arrays were scanned on an Affymetrix GeneChip scanner 3000 7G. Scanned arrays were analyzed first with Affymetrix Expression Console software for quality control. For subsequent analyses, Chipster 2.0 (http://chipster.csc.fi/) was used with custom cdf file HuGene10stv1_Hs_ENTREZG.cdf as available from Brainarray database version 14.1.0. GeneChip datasets for the arrays used in this study will be available in a MIAME-compliant format through Gene Expression Omnibus.

Validation by Real Time qPCR

Real-time quantitative PCR analysis (RT-qPCR) to confirm relative changes in mRNA levels of selected genes from microarray data sets was performed as described previously[82]. Briefly, reverse transcription of total RNA extracted was performed with

cDNA Synthesis kit (Quanta). RT-qPCR analysis was performed in a LightCycler 480 Multiwell Plate 96 (Roche), using the Light-Cycler 480 SYBR Green I Master Kit (Roche). cDNA was diluted 1:100 and each sample was analyzed in triplicate. The list of used primers and its sequence is presented in **Supplementary Table 1**. Cycles threshold (Ct's) and melting curves were determined using LightCycler 480 software, version 1.5 (Roche) and results were processed using the $2^{-\Delta\Delta Ct}$ method for relative gene expression analysis[82, 83]. Changes in gene expression were normalized using the house-keeping genes *HPRT1*, *GAPDH* and *B2M* as internal controls.

Functional enrichment and pathway analysis

Ingenuity® Pathway Analysis (IPA, Ingenuity Systems, Quiagen, USA) was used for functional enrichment analysis. To this end, we defined probe sets using remapped chip definition file (CDF) based on Entrez gene database. Changes in gene expression were assessed between BDP and medium control. The functional interpretation of gene signatures was executed using the core analysis function of Ingenuity Pathway Analysis (IPA) 3.0. IPA performs functional enrichment analysis based on up- and downregulated genes in order to identify the biological processes and functions over-represented in a given list of genes. In addition to this, based on information stored in the Ingenuity® Knowledge Base, upstream regulator analysis was performed using IPA software, which is based on the examination of the known targets of each transcription regulator in the list of differentially expressed genes, comparing their direction of change to what is expected from the literature. The prediction algorithm calculates a z-score (if ≥2 it is predicted to be activated, and if ≤-2 is predicted to be inhibited), and it is designed to reduce the chance that random data would generate significant predictions.

Statistical analysis

All results are averages of at least three independent experiments and are represented as the means ± SD. Differences amongst treatments were detected by analysis of variance with the Tukey HSD (honest significant difference) multiple comparison test (a = 0.05) using GraphPad Prism 6 software. Microarray raw data were normalized using Robust Multi-array average (RMA) and statistical differences evaluated by a two groups test (empirical Bayes), for $p<0.05$. Genes with significant changes ($p<0.05$ according

to Limma regularized paired *t*-test) were selected for IPA analysis. Significance of the enriched categories (Functional Enrichment Analysis of IPA) was tested by the Fisher Exact test *p*-value.

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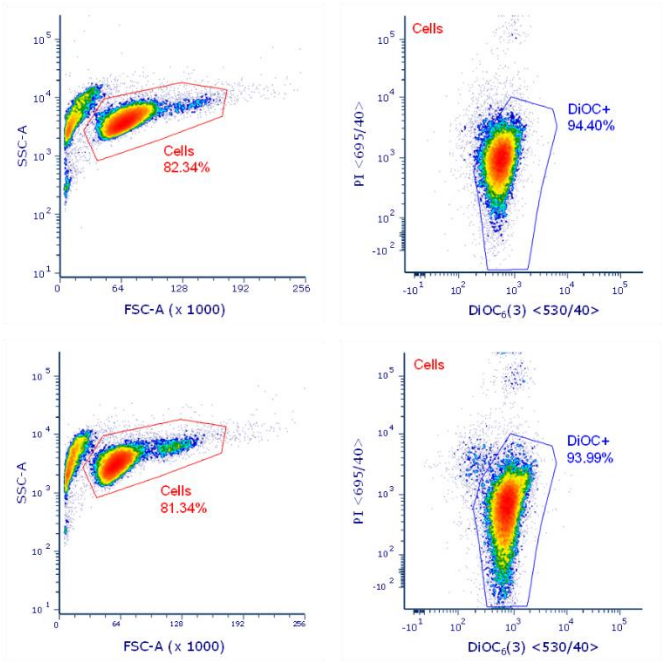
Supplementary Material

Supplementary Table 1 List of primers used in qRT-PCR validation.

Gene	Forward sequence	Reverse sequence
ESRP1	GAATGCTCAGGGTCGAAGGA	GGAGAGAAACTGGGCTACCTC
PPIL6	TTCAGCTGCCCCAACTTTCA	TGCCATGCAAATTCTTGAAGAGG
ALDH1L2	CTCCACTGGCCGGGTTTATT	AGCCAGAGGGTCAGCTTTTC
ASNS	ATGTTGGATGGTGTGTTTGC	AGTCGCGGAGTGCTTCAAT
ASS1	CAACACCCCTGACATTCTCG	TCTCGTAGATACCTCGGGACT
ATF5	ACTGGATGACTGAGCGAGTTG	GTCTTCCATCTGTTCCAGCTCC
PAG1	CGCAGTGGATACCATGCTCA	AGATTTTGCCTTGCCACTGT
NQO1	TGACATATAGCATTGGGCACAC	TTCTCCTCATCCTGTACCTCTT
SERPINF1	ATGATGTCGGACCCTAAGGC	TGCACGGTCTTCAGTTCTCG
SNORD56B	TCAACAGCGGTTCACCTAGT	TCACTCAAACCCAAAGTATCAACA
HPRT1	CCTGGCGTCGTGATTAGTGA	CGAGCAAGACGTTCAGTCCT
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC
B2M	GGCTATCCAGCGTACTCCAA	ACCAGTCCTTGCTGAAAGACAA



Supplementary Fig. 1 Representative picture of the isolation of SK-N-MC cells that presented cellular membrane integrity (PI negative) and high mitochondrial transmembrane potential ($DiOC_6(3)$ positive) using FACSAria High Speed Cell Sorter. SSC-A – side scatter, FSC-A – forward scatter. Top panels: control cells; Bottom panels: cells incubated with BDP.

Chapter 3

Bioavailable polyphenol metabolites are transported across the blood-brain barrier and modulate central hallmarks of neurodegeneration

This chapter is based on the following manuscript:

Polyphenols journey through blood-brain barrier towards neuronal protection
Figueira I, Garcia G, Pimpão RC, Terrasso AP, Costa I, Almeida AF, Tavares L, Pais TF, Pinto P, Ventura MR, Filipe A, McDougall GJ, Stewart D, Kim KS, Palmela I, Brites D, Brito MA, Brito C, Santos CN. (2017) *Scientific Reports* **7**, 11456. doi:10.1038/s41598-017-11512-6

This chapter contains data in which the author of this dissertation executed the majority of the experiments. Table 2 was performed by A.F. Almeida, Fig 3b was performed by I. Costa, Fig. 3c was performed by A.P. Terrasso and Fig 4 was performed by G. Garcia.

Table of contents

Abstract

Age-related complications such as neurodegenerative disorders are increasing and remain cureless. The possibility of altering the progression or the development of these multifactorial diseases through diet is an emerging and attractive approach with increasing experimental support. We examined the potential of known bioavailable phenolic sulfates, arising from colonic metabolism of berries, to influence hallmarks of neurodegenerative processes. *In silico* predictions and *in vitro* transport studies across blood-brain barrier endothelial cells, at circulating concentrations, provided evidence for differential transport, likely related to chemical structure. Moreover, endothelial metabolism of these phenolic sulfates produced a plethora of novel chemical entities with further potential bioactivities. Pre-conditioning with phenolic sulfates improved cellular responses to oxidative, excitotoxicity and inflammatory injuries and this attenuation of neuroinflammation was achieved *via* modulation of NF-κB pathway. Our results support the hypothesis that these small molecules, derived from dietary (poly)phenols may cross the BBB, reach brain cells, modulate microglia-mediated inflammation and exert neuroprotective effects, with potential for alleviation of neurodegenerative diseases.

Introduction

With increased life expectancy[1], the world's population is getting older with concomitant incidence of age-related diseases, like Alzheimer's and Parkinson's disease, the two most common age-related neurodegenerative disorders[2]. Current therapies only alleviate physical complications, being unable to abolish the pathology, comprising a huge burden to the society[3]. The difficulty in finding drugs to treat neurodegenerative disorders can be explained by the multitude of factors that lead to disease phenotype and effective treatments will need to be multi-faceted[4].

Through the past decades, several epidemiological studies have revealed that (poly)phenol-rich diets, including *e.g.* fruits and vegetables, can provide beneficial effects in humans[5, 6], preventing degenerative disorders and cognitive decline[7, 8]. (Poly)phenols are described as pleiotropic and may act against several disease-relevant biological pathways[9, 10]. Nutritional studies have also demonstrated significant cognitive benefits and neuroprotective potential of (poly)phenols[11-14]. Berries are amongst the most promising fruits as sources of (poly)phenols with these health benefits[11, 15-18].

Despite the accumulating evidence of beneficial effects, the basic mechanism of action of (poly)phenols remain to be elucidated[19].Both indirect actions through peripheral effects (*e.g.* enhancement of cerebrovascular blood flow) and direct actions inside the brain (*e.g.* through activation of receptors, neurotrophins and modulation of signaling pathways) have been suggested as potential mechanisms. Most *in vitro* mechanistic studies with (poly)phenols have used pure components and do not consider their metabolism and bioavailability. Therefore, the effects reported do not necessarily relate to what may occur *in vivo* as (poly)phenol metabolites present in circulation result from extensive conjugation due to digestion, hepatic and colonic metabolism, and usually differ from their native dietary compounds[20]. In addition, the concentration ranges used are much higher than the levels of circulating "bioavailable" metabolites.

Recent studies demonstrate that, after intestinal absorption, some (poly)phenol metabolites can reach concentrations in the bloodstream that can exert effects *in vivo*[21, 22]. Nevertheless, the effective brain uptake of these (poly)phenols metabolites, with possible direct neuroprotective potential, is still regarded with some reservations and

the true mechanisms by which they may permeate the blood-brain barrier (BBB) are not fully understood.

The BBB is a dynamic interface that limits and regulates molecular exchanges between the blood and the neuronal tissue or its fluid spaces, having a crucial role in providing nutrients and non-nutrients (such as (poly)phenols), and controlling the access of compounds to the brain[23, 24]. Assays with mammals revealed that (poly)phenols and their metabolites can enter the brain at measurable levels, supporting their direct action in a neurological context[25-27]. Youdim and co-workers have also begun to elucidate the mechanisms of permeation of (poly)phenols through the BBB[28, 29]. Nevertheless, it is not yet completely clear whether the primary route by which (poly)phenol metabolites cross the BBB is by simple diffusion or by specific carrier-mediated transport. Moreover, there is also limited knowledge of how (poly)phenol structure influences their brain bioavailability. Additionally, little is known about their further metabolism in the brain.

In previous work we identified new bioavailable (poly)phenol metabolites in urine and human plasma after consumption of a mixed berry puree[22, 30] and determined their circulating concentrations. These bioavailable metabolites circulate in micromolar concentrations whereas their parent compounds are undetected[22]. Here we report, for the first time, that these metabolites are able to cross the BBB endothelium at physiologically relevant concentrations. Moreover, endothelial cells metabolize these metabolites into novel components, which provides a new array of candidate brain-available metabolites never previously studied. We also demonstrate that these (poly)phenol metabolites exert beneficial effects in different neuronal systems (*e.g.* cell lines, primary cultures and a three-dimensional human cell model), with different degrees of complexity and in response to different damages. The (poly)phenol metabolites attenuated neuro-inflammatory processes *via* regulation of nuclear factor (NF)-κB translocation into the nucleus and modulation of IκBα levels.

Results

Bioavailable (poly)phenol metabolites are transported across the BBB endothelium

The transport of bioavailable (poly)phenol metabolites across the BBB was evaluated in an immortalized human brain microvascular endothelial cell (HBMEC) line that mimics endothelial cells of brain capillaries, considered the anatomical basis of the BBB[23, 31].

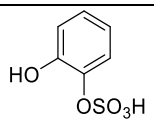
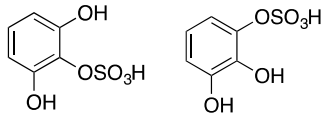
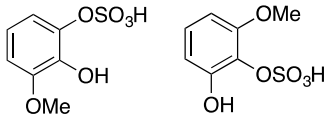
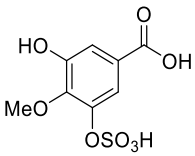
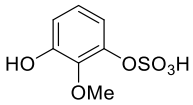
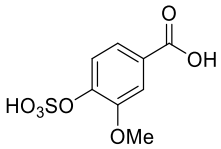
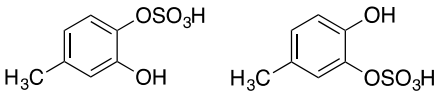
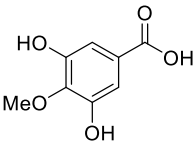
(Poly)phenol metabolites known to be bioavailable were synthetized and tested at 5 μM as they have been quantified at physiological levels ranging from 0.3-12 μM in plasma[22] (**Table 1**). At this range of concentrations, it was verified that there are no adverse effects on cellular viability in HBMEC line up to 24h of incubation for all compounds (**Supplementary Fig. S1**).

We used the well-validated confluent HBMEC two chamber BBB model[32] to investigate endothelium transport with metabolite quantification by Orbitrap LC-MS techniques. (Poly)phenol metabolites were added in the upper chamber and their putative transport through the BBB endothelium assessed after 2h of incubation (**Fig. 1a**).

Different concentrations of each compound were detected in the upper and lower chambers, suggesting a differential transport of the metabolites (**Fig. 1b**). For gallic acid (GA) derivatives, the combination of both methylation and sulfation, 4-*O*-methylgallic acid-3-*O*-sulfate (4-MeGA-sulf), increased BBB permeation compared with 4-methylgallic acid (4-MeGA). Intriguingly, 4-methylcatechol *O*-sulfate (4-MeCat-sulf) was more effectively transported than catechol-*O*-sulfate (Cat-sulf) (**Fig. 1b**). Moreover, in the case of pyrogallol-*O*-sulfate (Pyr-sulf), the position of the methylation influenced BBB passage; 1-*O*-methylpyrogallol-*O*-sulfate (1-MePyr-sulf) was less effectively transported whereas 2-*O*-methylpyrogallol-1-O-sulfate (2-MePyr-sulf) isomer was transported as effectively as Pyr-sulf. Additionally, Pyr-sulf consists in a mixture of isomers (Pyr-1-sulf and Pyr-2-sulf) (**Table 1**, see note 2) and we detected only Pyr-2-sulf in the basolateral compartment of cells.

No statistically significant difference was observed in the transport percentage when 5 or 10 μM of the metabolites was applied (**Supplementary Fig. S2**).

Table 1. Human bioavailable (poly)phenol metabolites. (Poly)phenol metabolites nomenclature, abbreviation, chemical structure and C_{max} are presented.

Nomenclature	Abbreviation	Structure	C_{max} (μM)[1]
Catechol-*O*-sulfate	Cat-sulf		12.2 ± 5.9
Pyrogallol-*O*-sulfate[2]	Pyr-sulf		11.4 ± 6.7 0.65 ± 0.3
1-*O*-methylpyrogallol-*O*-sulfate[3]	1-MePyr-sulf		2.88 ± 1.8
4-*O*-methylgallic acid-3-*O*-sulfate	4-MeGA-sulf		2.03 ± 1.1
2-*O*-methylpyrogallol-1-*O*-sulfate	2-MePyr-sulf		1.97 ± 1.0
Vanillic acid 4-*O*-sulfate	VA-sulf		1.34 ± 1.3
4-Methylcatechol *O*-sulfate[4]	4-MeCat-sulf		0.64 ± 0.5
4-*O*-methylgallic acid	4-MeGA		0.30 ± 0.1

[1] C_{max} values were determined by evaluation of human plasma samples in a previous work [22].

[2] mixture of two compounds in approximately similar proportion, 53% of Pyrogallol-2-*O*-sulfate (Pyr-2-sulf) and 47% of Pyrogallol-1-*O*-sulfate (Pyr-1-sulf), and for each of them C_{max} was obtained since they are chromatographically distinguishable.

[3] mixture of two compounds in approximately similar proportion, 56% of 1-*O*-methyl pyrogallol-2-*O*-sulfate (1-MePyr-2-sulf) and 44% of 1-*O*-methyl pyrogallol-3-*O*-sulfate (1-MePyr-3-sulf), chromatographically indistinguishable.

[4] mixture of two compounds (4-MeCat-1-sulf and 4-MeCat-2-sulf) present at 64% and at 36%, respectively, and chromatographically indistinguishable

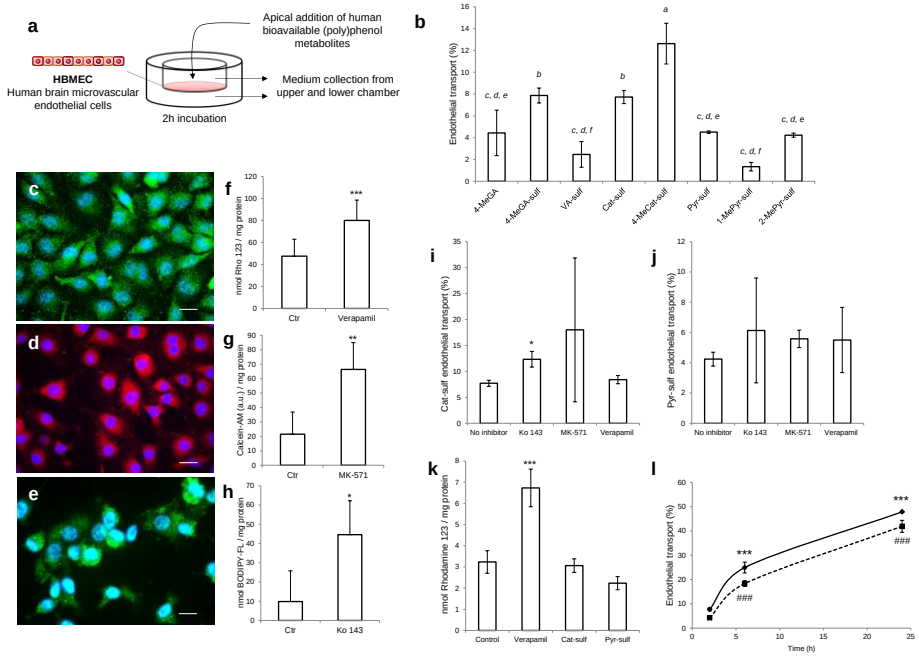


Fig. 1 Blood-brain barrier transport of human bioavailable (poly)phenol metabolites. (**a**) Schematic experimental design used to assess (poly)phenol metabolites transport across the BBB. (**b**) Endothelial transport of human bioavailable polyphenol metabolites after 2h of incubation. Endothelial transport was evaluated by LC-Orbitrap MS and is presented as percentage (%) determined by the ratio of the lower compartment concentration and the sum of the upper and lower compartments concentrations. Statistical differences for p<0.01 are denoted from *a-f*. (**c-e**) Immunofluorescence detection of major efflux transporters in HBMEC line: (**c**) P-gp, in green, (**d**) MRP1, in red and (**e**) BCRP, in green. Nuclei stained with DAPI (blue). Scale bar: 40 µm. (**f-h**) HBMEC intracellular accumulation of specific efflux transporters' substrates in the presence of the respective inhibitors: (**f**) 1µM of verapamil (P-gp inhibitor), (**g**) 1µM MK-571 (MRP1 inhibitor) or (**h**) 1µM of Ko 143 (BCRP inhibitor). Statistical differences are denoted as ***p<0.001, **p<0.01 and *p<0.05 relatively to control cells. Endothelial transport of (**i**) Cat-sulf and (**j**) Pyr-sulf when co-incubated with efflux transporters inhibitors. Statistical differences in the presence of inhibitors are denoted as *p<0.05 relatively to "No inhibitor". (**k**) P-gp substrate accumulation for Cat-sulf and Pyr-sulf compared with verapamil. Intracellular accumulation of P-gp substrate, Rhodamine 123 was evaluated after pre-incubation of cells with the bioavailable (poly)phenol metabolites. Statistical differences are denoted as ***p<0.001 relatively to control. (**l**) Endothelial transport of Cat-sulf (solid line) and Pyr-sulf (dashed line) along time. Statistical differences along time are denoted as ***p<0.001, relatively to 2h of incubation in Cat-sulf, or ### p<0.001, relatively to 2h of incubation in Pyr-sulf. All values are means ± SD, n=3.

In silico modeling of metabolite properties

Accessibility to the brain may be dependent to some extent on the structural properties of metabolites. An *in silico* prediction of blood-brain barrier permeability of the various metabolites was carried out using the QikProp software was carried out. QikProp predicts the ability of specific molecules to cross the BBB (**Table 2**). Estimated QikProp descriptors were within the range of values for 95% of known drugs (according to

Schrödinger software, https://www.schrodinger.com/qikprop) and did not vary much between the different compounds, which is reasonable considering their structural similarity. None of the compounds tested were predicted to have CNS activity only assuming passive BBB diffusion (CNS activity: -2).

For the octanol/water partition coefficient predictor (QPlogPo/w), the (poly)phenol metabolites were at the lower range of the recommended values, that indicates poor passive diffusion. Interestingly, the metabolites with a methyl group on (4-MeCat-1-sulf, 4-MeCat-2-sulf, 2-MePyr-sulf, 1-MePyr-2-sulf, 1-MePyr-3-sulf) gave the highest values (**Table 2**). Moreover, 4-MeCat-sulf and Cat-sulf gave the lowest PSA values (**Table 2**), which were below the recommended threshold of 90 $Å^2$, which also suggests that passive BBB permeation is more likely[33].

Another important descriptor that predicts the brain/blood partition is the QPlogBB. The calculated values for our molecules, although within the recommended values for brain drugs, were not high. Additionally, the predicted apparent MDCK cell permeability was very low for all compounds. The more charged compounds, such as 4-MeGA-sulf and VA-sulf have the lowest values (**Table 2**).

Table 2. *In silico* calculations of BBB permeation for human bioavailable (poly)phenol metabolites. QikProp descriptors were obtained for each metabolite, namely dipole, volume, donor HB, accptHB, QPlogPo/w, QPPCaco, QPlogBB, QPPMDCK, PSA, QPlogKhsa.

Molecule	Dipole	Volume (cm^3)	donorHB	accptHB	QPlogPo/w	QPPCaco (nm/s)	QPlogBB	QPPMDCK (nm/s)	PSA ($Å^2$)	QPlogKhsa
Cat-Sulf	8.5	541.2	2	5	-0.06	33.6	-1.24	16.6	89.4	-1.00
Pyr-2-sulf	10.5	549.2	3	6	-0.37	28.0	-1.38	13.6	96.7	-1.05
Pyr-1-sulf	9.4	586.8	3	6	-0.48	12.1	-1.70	5.5	110.9	-1.00
1-MePyr-2-sulf	5.9	627.0	2	6	0.24	48.4	-1.21	24.3	98.8	-0.97
1-MePyr-3-sulf	5.8	625.8	2	6	0.20	45.1	-1.23	22.5	100.1	-0.97
4-MeGA-sulf	4.8	676.8	2	8	-0.29	1.9	-1.90	0.9	128.4	-1.24
2-MePyr-sulf	2.9	635.1	2	6	0.25	43.3	-1.26	21.5	100.3	-0.96
VA-sulf	4.7	665.7	2	7	0.11	3.5	-1.73	1.8	114.1	-1.22
4-MeCat-1-sulf	9.0	625.8	2	5	0.34	33.5	-1.31	16.5	89.5	-0.85
4-MeCat-2-sulf	8.7	623.2	2	5	0.35	35.8	-1.27	17.7	89.5	-0.86
4-MeGA	2.9	573.4	3	4	0.23	34.7	-1.20	16.7	100.8	-0.85

donorHB – number of donor hydrogen bonds; accptHB – number of acceptor hydrogen bonds; QPlogPo/w – predicted octanol/water partition coefficient (for 95% of known drugs values range between -2.0 and 6.5); QPPCaco – predicted apparent Caco-2 cell permeability (non-active gut-blood barrier transport; <25 poor, >500 great); QPlogBB – predicted brain/blood partition coefficient (for 95% of known drugs values range between -3.0 and 1.2); QPPMDCK – predicted apparent MDCK (Madin-Darby Canine Kidney Epithelial Cells- consider a good model to mimic the BBB) cell permeability (non-active blood-brain barrier transport; <25 poor, >500 great); PSA – Van der Waals surface area of polar nitrogen and oxygen atoms and carbonyl carbon atoms (for 95% of known drugs values range between 7.0 and 200.0); QPlogKhsa – prediction of binding to human serum albumin (for 95% of known drugs values range between -1.5 and 1.5).

An important feature that could influence compounds ability to be actively transported through the BBB is their ability to establish hydrogen bonds with other functional groups. The carboxylic acids (VA-sulf, and 4-MeGA-sulf) have relatively high cumulative values as hydrogen exchangers (*e.g.* donorHB + accptHB). Moreover, the other carboxylic acid, 4-MeGA had highest value of QPlogKhsa, i.e. it is more likely to bind to human serum albumin. Overall, QikProp analysis suggested that none of the metabolites would be able to cross the BBB endothelium by passive permeation but some form of active transport could be involved.

HBMEC contains functionally active efflux transporters

Other factors could limit the levels of metabolites inside the brain besides transport mechanisms, such as efflux systems. To our knowledge, HBMEC cells have not yet been characterized for the expression or activity of major efflux transporters. By immunofluorescence, we detected the presence of the three major membrane ATP-binding cassette protein (ABC)-type efflux transporters in HBMEC cells (**Fig. 1c-e**), previously described to be present in brain endothelial cells and known for their broad substrate specificity; P-glycoprotein (P-gp, *ABCB1*), multidrug resistance-associated protein 1 (MRP1, *ABCC1*) and breast cancer resistance protein (BCRP, *ABCG2*)[34]. Functional activities of P-gp, MRP1 and BCRP were validated using substrate accumulation assays (**Fig. 1f-h**) and specific inhibitors for each transporter. Verapamil was used as a P-gp inhibitor and 1µM was sufficient to reduce its detoxifying capacity assessed by the intracellular accumulation of its substrate, Rhodamine 123 (**Fig. 1f**). We also confirmed the functional detoxifying activity of MRP1 (**Fig. 1g**) and BCRP (**Fig. 1h**), by using Calcein-AM and BODIPY-FL, respectively, as specific substrates, and MK-571 and Ko143 as the respective inhibitors.

Bioavailable (poly)phenol metabolites are not exported to the upper side and their transport increases with time

We also assessed how these efflux transporters could influence the permeation of (poly)phenol metabolites, in particular the most plasma-bioavailable metabolites Cat-sulf and Pyr-sulf[22], into the lower compartment of the BBB *in vitro* model (**Fig. 1a**).

Inhibition of the efflux transporters did not influence the BBB transport of Cat-sulf (**Fig. 1i**) and Pyr-sulf (**Fig. 1j**). However, BCRP may be partly involved in the efflux of Cat-sulf as there was a significant increase ($p<0.05$) in its endothelial transport after treatment with BCRP specific inhibitor, Ko 143 (**Fig. 1i**).

To determine if the metabolites could affect cellular efflux capacity, we evaluated the functional activity of the most well-studied efflux transporter in humans, P-gp[35]. Under the same conditions that Verapamil doubled Rhodamine 123 accumulation, no statistically- significant alterations in P-gp activity were noted in the presence of Cat-sulf and Pyr-sulf at 5 μM (**Fig. 1k**). The same trend was observed for the other bioavailable (poly)phenol metabolites (**Supplementary Fig. S3**). Transport of Cat-sulf and Pyr-sulf in the *in vitro* BBB model increased over 2, 6 and 24h. Although, there was a significant increase in transport percentage of both Cat-sulf and Pyr-sulf with time (**Fig. 1l**), even after 24h incubation, no transport of the less physiologically-representative isomer of Pyr-sulf (Pyr-1-sulf) was detected.

HBMEC metabolize bioavailable (poly)phenol metabolites

One phenomenon that may influence endothelium transport percentage for (poly)phenol metabolites across BBB is their metabolism within the cells. To identify possible cellular metabolites, the LC-MS data for the upper and lower compartments was searched against an in-house database of predicted metabolites[22] for compounds of predicted theoretical masses and fragmentation patterns and new putative cellular metabolites were detected. These included those arising from cellular conjugation with glucuronic acid or glutathione (**Supplementary Table S1**; results for the remaining metabolites are presented in **Supplementary Tables S2-4**).

The proposed cellular pathways of Cat-sulf and Pyr-sulf metabolism were designed based on canonical enzymatic reactions described in KEGG (Kyoto Encyclopedia of Genes and Genomes, **Fig. 2a**). Moreover, the relative abundance of the novel metabolites (**Fig. 2b-j**) detected in upper or lower compartments, was assessed, which indicated that each metabolite had a different pattern with time. Interestingly, we observed that the cellular metabolites more proximate to the original metabolites (*e.g.*

glutathionyl-pyrogallol, **Fig. 2b**) were detected in higher amounts than the ones more distant (*e.g.* acetylcysteine-pyrogallol, **Fig. 2c**).

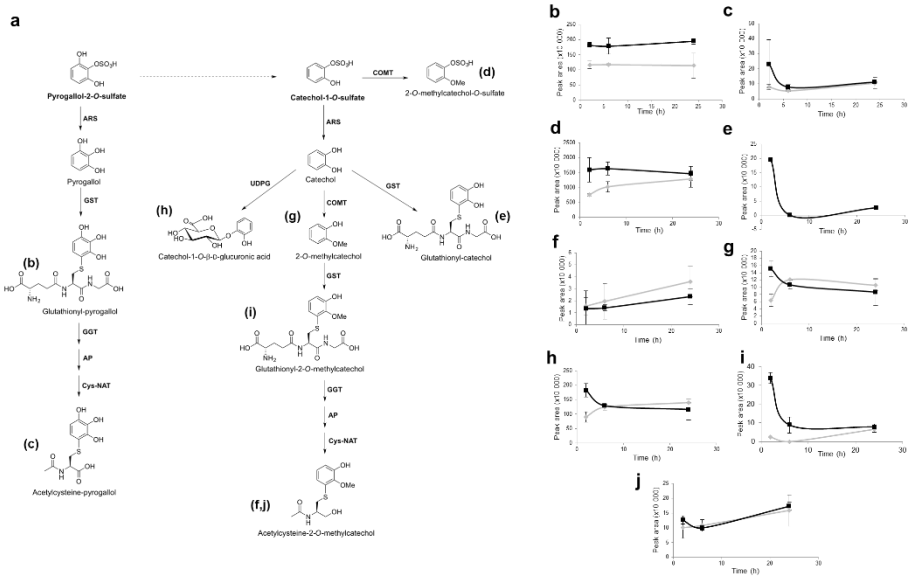


Fig. 2 Blood-brain barrier endothelial cells metabolization of Pyr-sulf and Cat-sulf. (**a**) Putative pathways and enzymes that could be involved in endothelial metabolization into novel phenolic compounds. Proposed metabolization route of the compounds was designed based on canonical enzymatic reactions described in KEGG (Kyoto Encyclopedia of Genes and Genomes). ARS -Arylsulfatase; GST - Glutathione S-transferase; GGT - Gamma glutamyl transferase; AP - Aminopeptidase; Cys-NAT – Cysteine N-acetyl transferase; COMT - Catechol *O*-methyl transferase; UDPG – UDP-Glucuronosyl transferase. Relative quantification (peak areas) of the novel phenolic metabolites appearing in upper (grey) and lower (black) compartments along time after addition of (**b-f**) Pyr-sulf or (**g-j**) Cat-sulf in upper compartment, namely (**b**) Glutathionyl-pyrogallol, (**c**) Acetylcysteine-pyrogallol, (**d**) 2-*O*-methylcatechol-*O*-sulfate, (**e**) Glutathionyl-catechol and (**f**) Acetylcysteine-2-O-methylcatechol, (**g**) 2-*O*-methylcatechol, (**h**) Catechol-1-*O*-β-D-glucoronic acid, (**i**) Glutathionyl-2-*O*-methylcatechol, and (**j**) Acetylcysteine-2-O-methylcatechol. Note: the compound Acetylcysteine-2-O-methylcatechol was detected in both samples (panels' **f** and **j**).

Bioavailable (poly)phenol metabolites protect brain endothelial cells and neuronal cells

Three different cell systems were selected to assess the breadth of neuroprotective potential of metabolites on neuronal excitotoxicity and oxidative stress, common hallmarks in neurodegenerative disorders[36]; a cell line representative/model of the BBB, primary cultures of mouse cerebellar granule cells, and human 3D aggregates of both neurons and astrocytes. The preventive effects of Cat-sulf and Pyr-sulf were assessed after pre-incubation at physiologically-relevant concentrations and prior to administering the specific insult.

As one of the first lines of defense, endothelial cells of the BBB prevent damage to the brain from constant insults[37]. The HBMEC line treated with 300 μM of hydrogen peroxide for 24h was used as a model of brain endothelial injury. Pre-incubation of Cat-sulf and Pyr-sulf prior to injury improved cell viability, with Pyr-sulf being more effective than Cat-sulf (**Fig. 3a**), maintaining cell viability at untreated cells levels. The other less abundant human bioavailable metabolites also caused significant cytoprotective effects to different extents (**Supplementary Fig. S4**).

Cat-sulf and Pyr-sulf were also assessed in a classic model of excitotoxicity[38], in which primary cultures of mouse cerebellar granule cells were challenged with glutamate. Both Cat-sulf and Pyr-sulf improved cell viability of cerebellar granular cells under glutamate-induced excitotoxicity (**Fig. 3b**), again with Pyr-sulf being more effective.

Pre-incubation of Cat-sulf and Pyr-sulf also caused significant neuroprotection in 3D aggregates against *tert*-butyl hydroperoxide (*t*-BHP)-induced injury (**Fig. 3c**). Both sulfates were equally protective and gave the same level of protection as conferred by idebenone[39], a drug used for of Alzheimer's disease known to boost mitochondrial ATP production[40]. Indeed, the other bioavailable metabolites also caused significant neuroprotective effects under the same conditions (**Supplementary Fig. S4**).

In all 3 cell systems, Pyr-sulf appeared to be more effective than Cat-sulf; therefore we decided to explore its mechanisms in greater depth using the 3D aggregates, a more physiologically-relevant system, and we compared it against the positive control idebenone[41]. Levels of βIII-tubulin and glial fibrillary acidic protein (GFAP) were studied as markers of neurons and astrocytes, respectively (**Fig. 3d-f**). The 3D aggregates challenged with *t*-BHP showed a significant decrease in βIII-tubulin protein levels but not GFAP protein levels, an effect counteracted by idebenone, which increased β-III tubulin protein levels. Pyr-sulf pre-incubation was ineffective in altering *t*-BHP induced changes in βIII-tubulin levels (**Fig. 3d-f**). Therefore, this suggests that *t*-BHP treatment has a greater impact on neuronal cells and this effect could not prevented by Pyr-sulf pre-treatment. Indeed, this cell-specific effect was hidden in the overall neuroprotective effect on total cell viability noted in **Fig. 3c**.

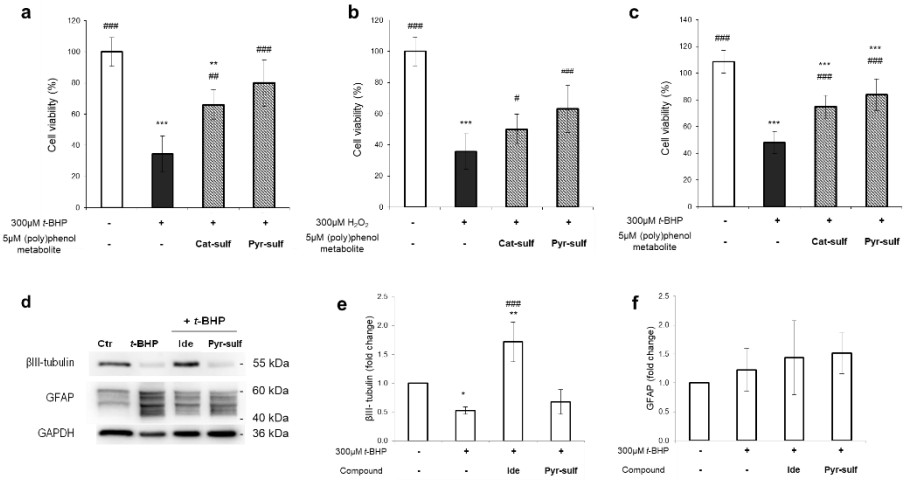


Fig. 3 Cytoprotective potential of Cat-sulf and Pyr-sulf. (**a**) HBMEC line submitted to oxidative stress (300µM H_2O_2); (**b)** primary mouse cerebellar granule cells exposed to glutamate excitotoxicity (100µM glutamate); (**c**) 3D aggregates containing neurons and astrocytes exposed to oxidative injury (300µM *t*-BHP). Cells were pre-incubated with 5µM of each bioavailable polyphenol metabolite for 24h and then injured with the respective lesion. Cell viability was assessed and is presented as percentage relatively to control. Statistical differences are denoted as ***p<0.001, **p<0.01 and *p<0.05 relatively to control and as ###p<0.001, ##p<0.01 and #<0.05 relatively to each lesion (H_2O_2, glutamate or *t*-BHP). (**d-f**) Alterations in protein markers of the neuronal (β-III tubulin) and astrocytic (GFAP) population of 3D aggregates. (**d**) Representative western blot and (**e**) β-III tubulin and (**f**) GFAP fold changes in protein levels normalized to GAPDH. Statistical differences are denoted as ***p<0.001, **p<0.01 and *p<0.05 relatively to control and as ###p<0.001 relatively to *t*-BHP. Western blots were analyzed under the same experimental conditions. Data are presented as the means ± SD, n=3.

After 24h of pre-incubation with Pyr-sulf before the *t*-BHP insult, expression of *SOD1*, *GPX1* and *GSR*, important antioxidant enzymes, were not significantly different from control, suggesting that the cells may already have returned to basal levels (**Supplementary Fig. S5**). Interestingly, *t*-BHP treatment alone significantly increased *SOD1* expression. We also did not see effects on the complex subunits from the mitochondrial respiratory chain that could be associated with Pyr-sulf protection (**Supplementary Fig. S5**). Finally we looked for key determinants in apoptosis. Pyr-sulf pre-incubation was not able to counteract the increased expression of the apoptosis regulator *BAX* gene. However it slightly increased the levels of the anti-apoptotic *BCL-2* (**Supplementary Fig. S5**).

Bioavailable (poly)phenol metabolites reduce neuro-inflammation

Neurodegenerative processes are also closely associated with neuro-inflammatory responses which are mainly mediated by the resident brain immune cells, microglia. Microglia pro-inflammatory activation was achieved by stimulation with lipopolysaccharide (LPS)[42] and the effect of pre-incubation with metabolites prior to LPS insult was studied (**Fig. 4** and **Supplementary Fig. S6**). We measured several parameters related to inflammatory responses such as reactive oxygen and nitrogen species (ROS/RNS), TNF-α inflammatory cytokine and CD40 expression. We found that pre-incubation with Pyr-sulf significantly inhibited TNF-α release (~2 fold) upon LPS stimulation (**Fig. 4a**) but no other markers of activation were affected by Pyr-sulf (**Fig. 4b**, **4c**, and **4d**). These results suggest that Pyr-sulf specifically affects TNF production. Nevertheless, some of the other less abundant bioavailable (poly)phenol metabolites also gave positive effects for the four pro-inflammatory markers, suggesting that each compound may have different potential (**Supplementary Fig. S6**).

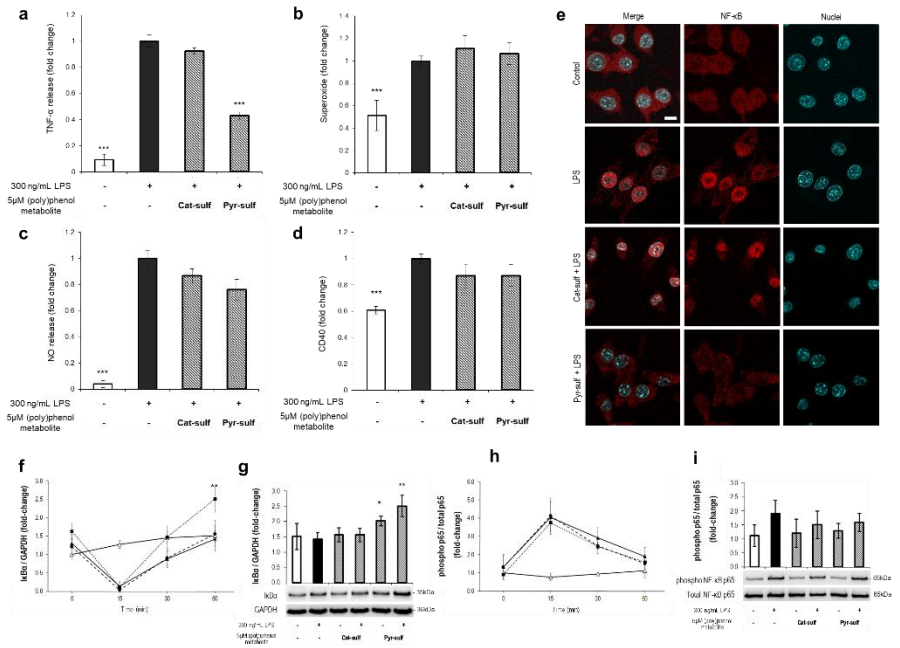


Figure 4 Effects on neuroinflammation by Cat-sulf and Pyr-sulf. Pro-inflammatory markers were evaluated, namely (**a**) TNF-α release, (**b**) nitric oxide, (**c**) intracellular superoxide production, and (**d**) CD40 quantified in N9 microglial cells. Cells were pre-incubated for 6h with each of the bioavailable (poly)phenol metabolites and then challenged with 300ng/mL of LPS. Statistical differences are denoted as ***p<0.001, **p<0.01 and *p<0.05 relatively to lesion (LPS). (**e**) Microglial NF-κB p65 translocation into the nucleus after 60 minutes of LPS stimulation. Cells were pre-treated with Cat-sulf or Pyr-sulf for 6 h before LPS-stimulation. NF-κB

(red); Nuclei (blue) stained with DAPI. Each capture is representative of at least 3 independent biological replicates. Scale bar: 10 μm. (**f-i**) Microglial NF-κB p65 phosphorylation ratio and IκBα fold change in protein levels. (**f**) IkBα protein levels along time after LPS stimulation. and (**g**) IkBα fold change in protein levels after 60 min of LPS stimulation and representative western blots. (**h**) NF-κB activation profile along time after LPS stimulation looking at NF-κB p65 phosphorylation (ser536) ratio and (**i**) NF-κB p65 phosphorylation ratio Cells were pre-treated either with Pyr-sulf or Cat-sulf before LPS stimulation. Control cells (white triangles, solid line), LPS-stimulated cells (black triangles, solid line), cells treated with Cat-sulf prior to LPS stimulation (black circles, dashed line), cells treated with Pyr-sulf prior to LPS stimulation (black squares, dotted line). Statistical differences are denoted as *p<0.05 and **p<0.01 relatively to lesion (LPS). Western blots were analyzed under the same experimental conditions. Data are presented as the means ± SD, n=3.

Pyr-sulf prevents NF-κB nuclear translocation and alters IκBα levels

TNF-α levels are mainly regulated by the NF-κB pathway[43]. To investigate the mechanisms underlying the inhibitory effect of Pyr-sulf on TNF production, we analyzed NF-κB activation. We followed NF-κB translocation to the nucleus and its phosphorylation levels on ser536. In addition, we analyzed the kinetics of IκBα degradation, which inhibits NF-κB translocation to the nucleus[43].

After one hour, LPS induced a clear nuclear translocation of NF-κB to the nucleus of N9 cells (**Fig. 4e**). However, pre-incubation with Pyr-sulf but not Cat-sulf prevented LPS-stimulated NF-κB translocation to the nucleus in microglia. Similar results were obtained with primary cultures of rat microglia (**Supplementary Fig. S7**). N9 cells treated with LPS showed, as expected, a significant decrease in IκBα levels after 15 min when compared to non-treated cells (**Fig. 4f**). Pre-incubation with Pyr-sulf did not prevent IκBα reduction at 15 min, but it boosted the recovery of IκBα levels after 30-60 min of LPS treatment (**Fig. 4f** and **4g**). Interestingly, Pyr-sulf alone enhanced IκBα basal levels, even without an inflammatory stimulus (**Fig. 4g** and **Supplementary Fig. S8**). The NF-κB phosphorylated-p65/total p65 ratio peaked at 15 min (**Fig. 4h)** and then decreased to basal cellular levels at 60 min (**Fig. 4h** and **4i**). This kinetics was unchanged by pre-treating either with Pyr-sulf or Cat-sulf (**Supplementary Fig. S8**). Overall, our data suggest that Pyr-sulf pretreatment increased the stability of IκBα rather than inhibiting NF-κB phosphorylation.

Discussion

Dietary (poly)phenols present neuroprotective potential but their selective permeability across BBB, poor absorption, rapid metabolism and systemic elimination limit their bioavailability and could limit their protective efficacy[21, 27].

Taking advantage of a robust, though simplified, *in vitro* model of the human BBB which has been validated for CNS drugs[32, 44, 45], we confirmed that plasma-bioavailable (poly)phenol metabolites could be transported across the BBB endothelium. The differences in endothelial transport for the metabolites could be related with their degree of chemical modification: methylation combined with sulfation enhanced the transport of gallic acid and catechol derivatives, but the same was not true for pyrogallol derivatives. In fact, Youdim and colleagues noted that transmembrane diffusion of some (poly)phenol metabolites *in vitro* was related to their lipophilicity, where less polar (e.g. methylated) derivatives achieved greater uptake than more polar derivatives (e.g. sulfates and glucuronides)[28, 29]. Nevertheless, it is not clear whether the primary route by which (poly)phenols metabolites cross the BBB is by simple diffusion or by carrier-mediated transport.

Our data for BBB endothelial transport can be partially explained by the *in silico* predictions. For instance, 4-MeCat-sulf, the metabolite with the higher endothelial transport percentage, also presented the higher QPlogPo/w. Moreover, for this metabolite and for Cat-sulf, the lowest PSA values were reported, below the recommended threshold of 90 $Å^2$ to be considered suitable for passive BBB permeation[33]. It is important to highlight that the descriptors calculated by the *in silico* analysis have to be regarded as whole and are integrated only assuming passive BBB diffusion. However, the contribution of active transport and metabolic transformation should not be discounted. In fact, the chemical ability of some metabolites to establish hydrogen bonds (indicated by some descriptors) with other functional groups of biomolecules (e.g. amine and hydroxyl groups of proteins like albumin) could suggest a propensity for an active transport mechanism in BBB. Nevertheless, such descriptors do not necessarily imply that the studied molecules are inactive in the brain, as there are other ways to transport molecules across the BBB, as it is seen for very polar essential molecules such as hexoses, amino acids and several orally administered

medicines and other drugs. Overall our experimental results, together with the *in silico* predictions, suggest that transport across BBB could not be simply justified by passive permeation and some form of active transport must also be involved.

Interestingly, HBMEC cells seemed to favor the transport of one isomer of Pyr-sulf, Pyr-2-Sulf, which was also the main form found in human plasma[22, 30]. Even after an extended incubation period (24 h), no transport of the other isomer, Pyr-1-sulf was detected, suggesting selective transport. This may be due to the enhanced hydrogen bonding ability of the vicinal diol structure in Pyr-2-sulf compared to Pyr-1-sulf which could influence protein binding[46]. Existence of an isomer-selective transport in endothelial cells of BBB has already been reported for (+)-catechin and (-)-epicatechin[47], although this is a stereoisomer rather than a positional isomer. Our results suggest that HBMEC may favor the uptake of the more bioavailable metabolite of a mixture in equal proportions.

The presence of efflux transporters was confirmed for the first time in HBMEC cells and these ABC-type transporters may contribute to overall BBB transport of the (poly)phenol metabolites. There was a slight but significant increase in Cat-sulf transport in the presence of a BCRP inhibitor, which suggests that this efflux transporter can limit Cat-sulf transport. In fact, other studies have also highlighted the involvement of BCRP in limiting (poly)phenol access to the brain. For instance, quercetin, when co-administrated with a P-gp or BCRP inhibitor, entered BBB epithelia but was then specifically exported by the BCRP[28]. Such phenomenon may partly explain why quercetin presents very limited bioavailability, despite its plasma bioavailability.

As well as acting as substrates for these efflux pumps, (poly)phenol metabolites can modulate the activity of ABC transporters, putatively influencing brain bioavailability of other compounds (reviewed in [48]). However, our results for P-gp activity suggest that none of the metabolites activated or inhibited this efflux pump. Therefore we propose that differential transport observed across the BBB endothelium cannot be a consequence of a differential efflux of the (poly)phenol metabolites. It should be noted that the BBB is a complex interface composed not only by endothelial cells but also by astrocytes and perycites[23, 31], and these cell types may also influence BBB transport of (poly)phenol metabolites *in vivo*.

Taking advantage of the power of the MS analysis, we observed that HBMEC metabolized the phenolic sulfates into novel metabolites, mainly glutathione and glucuronic acid derivatives. Indeed, capillary endothelial cells are known to possess the enzymes required for these conversions, such as glutathione S-transferase, UDP-glucuronosyltransferase[49], gamma glutamyl transpeptidase and catechol-*O*-methyltransferase[49]. Enzymes known primarily for hepatic drug metabolism have also been shown to exist in the brain, albeit at relatively low specific activities[50] in particular at the blood-brain interfaces where they influence cerebral availability of toxic compounds[49, 51]. An *in vitro* study to assess (+)-catechin and (-)-epicatechin permeability across the BBB detected new glucuronide derivatives using the human brain capillary endothelial cell line hCMEC/D3[47]. Similarly, Liang and co-workers[52] performed a comprehensive LC-MS study on (+)-catechin metabolism product distribution in rat tissues and reported 3-*O*-Me-catechin-5-*O*-glucoronide was present in the brain tissue. Our studies also provided evidence for the interconversion of some metabolites into new metabolites. For example, acetylcysteine-2-O-methylcatechol, was detected in both Cat-sulf and Pyr-sulf samples, although in different relative amounts. Also, novel metabolites were detected in samples from gallic acid derivatives and from VA-sulf. The extensive metabolism of these simple phenolic sulfates by HBMEC reinforces the importance of studying the metabolites of (poly)phenols and not their parent compounds. We speculate that further interconversions may occur to, for example, valeric acid and hydroxyphenyl-propanol derivatives [52] and the physiological relevance of these new metabolites is unknown. Moreover, other cell types resident in the BBB besides endothelial cells, may also contribute to the generation of different new (poly)phenol variants. Further studies with more complex BBB *in vitro* models (*e.g.* co-cultures of brain endothelial cells with astrocytes) will better elucidate potentially novel brain-targeted (poly)phenol metabolites. On the other hand, by contributing also for the synaptic function, astrocytes role in (poly)phenol metabolites supply to neurons in a neuronal *in vivo* context must be further contemplated.

The (poly)phenol metabolites caused a cytoprotective effect in the BBB cells challenged with hydrogen peroxide. Pyr-sulf, the most abundant metabolite detected in circulation[22], was the most effective to prevent oxidative damage caused in HBMEC cells by

hydrogen peroxide. BBB endothelial protection by resveratrol has been described[37] but, to our knowledge, this is the first time that protection has been demonstrated using physiological concentrations of bioavailable (poly)phenol metabolites.

Neuroprotective effects of dietary (poly)phenols in primary neuronal cultures and in a 3D model containing neurons and astrocytes was also observed. In glutamate excitotoxicity conditions, pathologically relevant for neurodegenerative disorders, neurons are damaged by the excessive stimulation of nerve receptors through this neurotransmitter accumulation in the synaptic cleft[53]. Pyr-sulf caused significant neuroprotection in primary mouse cerebellar granule cells exposed to toxic glutamate concentrations. A similar protection was recently noted for pterostilbene, a resveratrol analog, described to be brain bioavailable, which attenuated glutamate-induced oxidative stress injury in murine hippocampal neuronal HT22 cells at physiological circulating levels[54].

The 3D aggregates are a very robust system, with different functional cell types (neurons and astrocytes), interlinked and communicating, in a three-dimensional architecture, which is more directly related with *in vivo* environment of brain cells[41, 55]. The neuroprotection by human bioavailable (poly)phenol metabolites in these neuron-astrocyte models reinforces the pharmacological importance of these metabolites. Exposure to *t*-BHP induced greater damage to neurons than astrocytes within the 3D aggregates but Pyr-sulf did not recover the levels of the neuronal marker, βIII-tubulin and also did not affect GFAP protein levels (the astrocyte marker). Neurons have been described to have a greater susceptibility to oxidative injury as compared to astrocytes[56]. In fact, our 3D aggregates are mainly composed of astrocytes (~77% of the cells)[41]. Glial cells (astrocytes and microglia) are responsible for the repair process of the brain after injury[57], being stronger and less susceptible to damage than neurons. Protection by Pyr-sulf in this system could then be related with the improving functionality of astrocytic population inside aggregates and not directly reducing the deleterious effects of the oxidative injury. However, there is still a lack of studies demonstrating the effect of phenolic metabolites on these cells. It have already been described that resveratrol can ameliorate glutamatergic metabolism and transmission and thus synaptic plasticity and neuroprotection[58-60]. The missing link between (poly)phenol metabolites effects and

neuroglial communication/signaling is therefore of upmost importance to be addressed for an effective translation of how diet can alter age-related neurological diseases, like Alzheimer's disease.

Inflammation and neurodegeneration are normally associated in neurological conditions. Here, we found that, Pyr-sulf, besides its neuroprotective effect, also attenuated TNF-α release in microglia challenged with LPS. TNF-α, a classic and reliable marker of microglia activation, is known to be regulated by NF-κB. Dietary phenolics, such as quercetin, curcumin and resveratrol, have been shown to inhibit signaling pathways involved in microglia cells activation[61], namely NF-B. However, the effect of their metabolites has not yet been assessed probably due to their poor bioavailability. To our knowledge, this is the first study where dietary (poly)phenol metabolites have been shown to modulate NF-κB-mediated microglia activation. Our results show that Pyr-sulf, at physiological concentrations, is able to modulate IκBα protein levels, either by promoting its synthesis or by decreasing its degradation, which retains NF-κB in the cytoplasm and prevents NF-κB-dependent gene transcription. Indeed, this could explain the reduction in TNF-α levels in LPS-stimulated microglia pre-incubated with Pyr-sulf.

Taken together, these data provide new insights for the (poly)phenol metabolites to be further explored in biochemical pathways and validated *in vivo* using appropriate animal models. Although (poly)phenol metabolites could impact on brain health and cognition indirectly through peripheral and cerebrovascular blood flow improvement, this work highlights their potential to have direct effects towards neuronal cells. The phenolic sulfate metabolites studied are transported across endothelial cells of the BBB and metabolized to form new chemical entities with their own potential biological effects. While we cannot predict the biological role/relevance of these biotransformations, the fact that BBB cells modify (poly)phenols metabolites is of vital importance for further studies. These biotransformation processes by HBMEC may facilitate metabolite elimination from brain or, on the other hand, such modifications may enhance retention or assist further uptake and delivery to other neuronal cell types, therefore spreading their beneficial effects. Moreover, since the BBB is the first line of defense of the brain

and is a crucial preventive factor of neurological diseases, ensuring BBB endothelium protection through diet could have wide-spread consequences in the body. The neuroprotective potential of these phenolic sulfates, used at physiologically relevant concentrations, comparable to those detected in human plasma[22], reflects their importance as modulators of cell metabolism and the ultimate importance of dietary habits to health and disease progression. Moreover, our studies on the abundant serum-bioavailable metabolite, Pyr-sulf, emphasized the potential pleiotropic neuroprotective effects of these phenolic metabolites. The marked reduction in TNF-α release may be associated with the neuroprotective power of Pyr-sulf: the capacity of Pyr-sulf to pre-condition cells, preventing NF-κB nuclear translocation and being able to better respond to oxidative stimuli, an excitotoxicity burst or an inflammatory situation, confirms the potential of these human bioavailable (poly)phenol metabolites to mitigate in the intricate complexity of a neurodegenerative disorder.

Material and Methods

Reagents

All the used chemicals were purchased from Sigma-Aldrich, unless stated otherwise. Acetonitrile (ACN, LC-MS grade) was purchased from Fisher Scientific Ltd. (Leicestershire, UK). LC-MS grade water was produced by an Elix/MilliQ purification system (Millipore, Waterford, UK). 4-Methylgallic acid and 2-methylpyrogallol were obtained from Apin chemicals and taxifolin was obtained from Extrasynthese. The synthesized compounds were: 4-methylgallic acid-3-*O*-sulfate (4-MeGA-sulf, 57% yield, 79% purity, containing 6% of 4-methylgallic acid and 13% of 4-methylgallic acid-3,5-*O*-disulfate), 4-methylcatechol-*O*-sulfate (4-MeCat-sulf, 66% yield, mixture of two compounds indistinguishable, pure), vanillic acid-4-*O*-sulfate (VA-sulf, quantitative yield, pure), catechol-*O*-sulfate (Cat-sulf, 66% yield, pure), pyrogallol-*O*-sulfate (Pyr-sulf, 75% yield, mixture of two compounds in equal proportions), 1-methylpyrogallol-*O*-sulfate (1-MePyr-sulf, mixture of two compounds in equal proportions, 58% yield) and 2-methylpyrogallol-*O*-sulfate (2-MePyr-sulf, 44% yield, 89% purity, containing 11% of 2-methylpyrogallol-*O*-disulfate). Synthesized compounds were obtained as sodium salts and were firstly dissolved in DMSO (Fluka) before dilution to final concentration in specific cell media (see **Table 1**).

Cell culture conditions

HBMEC line

Human brain microvascular endothelial cell (HBMEC) line was used as a simplified and validated *in vitro* model of the BBB[32, 44, 45, 62]. This cell line was derived from primary cultures of HBMEC transfected with SV40 large T antigen[63]. HBMEC line was cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS - Biochrom AG), 10% NuSerum IV (BD Biosciences), 1% non-essential amino acids (NEAA - Biochrom AG), 1% minimal essential medium (MEM) vitamins (Biochrom AG), 1mM sodium pyruvate (Biochrom AG), 2mM L-glutamine (Biochrom AG), and 1% antibiotic-antimycotic solution (Sigma-Aldrich). For immunostaining and cytoprotection

studies, cells were seeded at a density of 8×10^4 cell/mL in 24-well and 96-well plates, respectively, and treated after 48h in culture. For integrity and transport studies, cells were seeded on polyester transwell inserts (0.4 µm, Corning Costar Corp., USA) at a density of 8×10^4 cell/insert and treated after 8 days in culture. Inserts and plates were coated with rat-tail collagen-I (BD Biosciences, Erembodegem, Belgium) before seeding. All experiments were performed after monolayer formation.

Mouse cerebellar granular cells

Primary cultures of cerebellar granule cells were prepared according to the already described method[38]. Cells were isolated from cerebella of 7d-old BALB-C mice and 0.5 x 10^6 cell/mL were cultured in Neurobasal Medium (Gibco) supplemented with 2% B-27 without antioxidants (Gibco) and 2% KCl (20mM, Sigma-Aldrich), containing 0.25% L-glutamine (200 mM, Sigma-Aldrich) and 0.48% penicillin-streptomycin (P/S, 5000 U mL^{-1}, Gibco). Experiments were performed on 24-well plates with coverslips coated with 50 µg/mL poly-D-lysine (Sigma-Aldrich). 20 µM of cytosine arabinoside (Sigma-Aldrich) was added 48 h after inoculation to prevent glia cell proliferation. All the experiments were performed between days 7-11 in culture.

N9 murine microglial cell line

The N9 murine microglial cell line was kindly provided by Dr. Teresa Faria Pais. Cells were cultured in EMEM (Eagle Minimum Essential Media, Sigma-Aldrich) supplemented with 10% FBS (Gibco), 200 mM L-glutamine (Sigma-Aldrich), 1% NEAA (Sigma-Aldrich) and maintained at 37°C, 5% CO_2. Cells were detached by agitation before suspension of the culture media with a pipette (no cellular detaching agent was used).

NT2 cell line

NTera-2/cl.D1 (NT2) cells, obtained from American Type Cell Culture Collection (ATCC), were differentiated in stirred suspension culture systems, as 3D aggregates, as previously described[41]. A single cell suspension of undifferentiated NT2 cells was seeded in DMEM (Dulbeco Minimum Essential Media), 10% FBS, 1% P/S (all from Invitrogen), in a 125 mL spinner vessel equipped with ball impeller (Wheaton). After

three days of aggregation, differentiation was induced by addition of 10 μM retinoic acid (Sigma-Aldrich), for three weeks, with a 50% medium exchange performed every 2-3 days. Following this period (from day 24 onwards), the medium was composed by DMEM, 5% FBS, 1% P/S. Stable 3D co-cultures of neurons and astrocytes were maintained up to day 50 of culture and applied in neuroprotection assays (from day 38 to day 50).

Immunofluorescence

Both HBMEC and N9 cells were grown in 24-well plates with coated coverslips and immunostaining performed as already described[62, 64]. Briefly, HBMEC coverslips were incubated overnight at 4°C with primary antibodies anti-P-gp (1:50, Calbiochem), anti-MRP1 (1:100, Millipore) and anti-BCRP (1:100, Millipore) and N9 cells coverslips were incubated overnight at 4°C with rabbit polyclonal anti-NF-κB p65 (C-20) (1:200, Santa Cruz Biotechnology). Incubation with secondary antibodies Alexa 594 anti-rabbit IgG (1:500) and Alexa 488 anti-mouse IgG (1:500) (Invitrogen) lasted for 2h at room temperature. Nuclei were counterstained with DAPI. Between incubations cells were washed three times with PBS. HBMEC staining was examined using a Leica DFC 490 camera (Leica, Germany) adapted to an AxioScope.A1 microscope (Zeiss, Germany), ZEN 2012 blue edition software by Carl Zeiss Microscopy GmbH, 2011. Confocal fluorescent Z-series N9 cells were acquired using on a Leica SP5 live upright confocal (Leica, Wetzlar), using a 63x 1.3NA oil immersion objective, the UV lamp and DPSS 561nm yellow-green laser. Post-acquiring treatment was performed using ImageJ software (NIH, USA).

Efflux transporters functional assays

Activity of each efflux transporter was determined by measuring cellular accumulation of respective substrate[65, 66], and results were expressed as fold-change as compared to the respective control. Activity of P-gp, MRP1 and BCRP were determined by measuring the cellular accumulation of the substrates Rhodamine 123 (Sigma-Aldrich), Calcein-AM (Santa Cruz Biotechnology), and BODIPY-FL Prazosin (Life Technologies), respectively. HBMEC monolayers were washed and incubated for 1 h at 37 °C with

Ringer–Hepes solution (118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.5 mM D-glucose, 20 mM Hepes, pH 7.4) containing 10 mM of each substrate, separately. The solution was quickly removed, HBMEC were washed three times with PBS and solubilized in 0.1 M NaOH. Substrate content was determined using a FLUOstar Omega fluorescent plate reader (BMG Labtechnologies, Ortenberg, Germany; for P-gp, excitation at 505 nm, emission at 534 nm; for MRP1, excitation at 495 nm, emission at 516 nm; for BCRP excitation at 503 nm, emission at 512 nm). A reference efflux transporter inhibitor was used as positive control: P-gp inhibitor, Verapamil (1µM, Sigma-Aldrich); MRP1 inhibitor, MK-571 (1 µM, Santa Cruz Biotechnology); and BCRP inhibitor, Ko 143 (1 µM, Santa Cruz Biotechnology). Protein content was evaluated by the Bradford method [67] using Bio-Rad's Protein Assay reagent (Bio-Rad).

BBB integrity

Trans-endothelial electrical resistance (TEER)

TEER was evaluated as reported previously[62]. Briefly, TEER readings were performed using an End Ohm™ chamber coupled to an EVOMX resistance meter (World Precision Instruments, Inc., USA). Readings were collected before the addition of bioavailable (poly)phenol metabolites and at the end of the incubation time. TEER was calculated as percentage of variation from average control readings, after deducting the empty insert values.

Paracellular permeability

To evaluate selective paracellular permeability of the HBMEC monolayer after exposure to the bioavailable (poly)phenol metabolites, a permeability assay was conducted with sodium fluorescein (molecular weight: 376 Da). The permeability was determined as described before[62]. The endothelial permeability coefficient *Pe* was calculated as described[68] as a percentage of variation from control. In all the assays, monolayer integrity was monitored by TEER and sodium-fluorescein paracellular permeability, which confirmed that the passage of metabolites across BBB cells was not due to a disruption of the barrier properties.

Transport Assays

HBMEC were plated in semi-permeable membranes (inserts) placed in cell culture wells. This two-chamber system, where the upper and lower chambers mimic the blood and brain compartments, respectively, and the confluent HBMEC monolayer represents the BBB. Transport assays were conducted in HBSS (Hank's Balanced Salt Solution) with calcium and magnesium (Gibco), supplemented with 0.1% FBS. Confluent monolayers of the HBMEC were incubated with 5 µM of each compound for 2h, time described for compounds to interact and/or being transported across cells[26, 69]. In order to evaluate a time-dependent transport, 5 µM of Cat-sulf and Pyr-sulf were added to the upper site and samples from upper and lower site were collected after 2, 6 and 24h. To evaluate efflux transporters influence in the BBB transport of the bioavailable (poly)phenol metabolites, co-incubation of 5 µM of Cat-sulf and Pyr-sulf with inhibitors of P-gp, (1 µM Verapamil), of MRP1 (1 µM MK-147) and of BCRP (1 µM Ko 143) was also performed. Monolayer integrity was ensured in all experiments as described. In the end, cell medium from upper and lower compartments were collected and frozen at -80 °C until analysis. Transport analysis were performed after sample deproteinization by LC-Orbitrap MS as described below.

Sample deproteinization

To 1 mL of upper or lower compartment cell medium it was added: 139 µL of 50% formic acid, ascorbic acid (final concentration of 1 mM) and taxifolin as internal standard (final concentration of 9 µM). To precipitate proteins, 2.5 mL of ACN was added dropwise and samples were vortexed before centrifugation at 3200 *g* for 15 min. The supernatant was removed and dried under centrifugal evaporation (CentriVap Vaccum Concentrator, Labconco). Samples were dissolved in 5% ACN in 0.1% formic acid and immediately analyzed by LC-Orbitrap MS.

LC-Orbitrap MS

Samples were separated on an HPLC Accela 600 HPLC system (Thermo Scientific, Bremen, Germany) using a C18 Synergi Hydro RP18 column (Phenomenex, Macclesfield, UK) 4 µm particle size and dimensions 2 mm ID × 150 mm. Column was

fitted with a Security GuardTM guard system containing an Aqua 10 µm C18 Guard Cartridge (2mm ID×4 mm; Phenomenex) and eluted over a gradient of 98% solvent A (0.1% formic acid in ultra-pure water) to reach 5% B (0.1% formic acid in ACN) at 5 min, 35% B at 25min, increase to 100% B at 26 min, 100% B at 29 min, and back to 2% B at 30 min at a flow rate of 0.2 mL/min. Analysis was done on an LTQ OrbitrapTM XL hybrid mass spectrometer (Thermo Scientific, Bremen, Germany).

MS analysis was performed using data-dependent *N*th order double play analysis comprising full scan mass range 80–2000 amu, 30 000 resolution, data-type centroid and data dependent MS/MS (60 s of exclusion duration) on the top three most intense ions detected above threshold automatically in the independent scan event. ESI settings were as follows: source voltage, 3.4 kV; capillary temperature was 275°C with a sheath gas at 40 psi and auxiliary gas at 5 psi. MS data handling software (Xcalibur QualBrowser software, Thermo Electron Corp.) was used to search for predicted metabolites by their appropriate *m/z* value. All peaks were checked for *m/z* value and fragmentation products. Calibration curves, ranging from 0.3125 to 10µM, were constructed from all the metabolites, and each concentration point was injected in triplicate. Standard curves were all linear within the concentration range and linearity was ensured as R^2 0·997–1·000. Limit of quantification was determined by analysis in triplicate of standards at low concentrations, and was defined as signal: noise ratios of 1:10. Endothelial transport was calculated as percentage determined by the ratio of lower compartment concentration and the sum of upper and lower compartments concentrations.

Search for novel compounds resulting from HBMEC metabolization was performed in the same samples using Xcalibur QualBrowser software using an in-house database of putative bioavailable (poly)phenol metabolites[30]. Putative ID of predicted metabolites was determined by exact mass, according to their appropriate *m/z* value.

In silico prediction of pharmacokinetic properties

Maestro software (Schrödinger, Release 2015-4, LLC, New York, 2014) was used to create a three-dimensional computer models of studied compounds. The global minimum geometry was used as an input for the QikProp application (Schrödinger Release 2015-4, LLC, New York, 2014) to estimate various theoretical descriptors relevant for compound permeability through the BBB[70]. From QikProp pharmaceutically-relevant properties, the following parameters were retained for evaluation: octanol/water partition coefficient; predicted brain/blood partition coefficient; apparent Caco-2 or MDCK cell permeability; predicted CNS activity; Van der Waals surface area of polar nitrogen and oxygen atoms and carbonyl carbon atoms (PSA); prediction of binding to human serum albumin.

Protective potential of human bioavailable (poly)phenol metabolites

Cytoprotection in HBMEC

HBMEC were incubated in the presence of H_2O_2 (Sigma-Aldrich). Briefly, 24 h after seeding, cells growth medium was removed and cells were pre-incubated for 24h with 5 µM of each compound. After pre-incubation, media was replaced by new media containing 300 µM H_2O_2, for 24 h[71, 72]. In the end, medium was removed and cell viability was assessed using the CellTiter-Blue® Cell Viability Assay (Promega), according to the manufacturer's instructions.

Neuroprotection in cerebellar granule cells

Physiological concentrations (5 µM) of the two most abundant metabolites were added to the cells at 7d in culture. Cells were then incubated for 24 h and morphology was evaluated. At day 8 in culture, 100 µM of glutamate (Sigma-Aldrich) was added to the respective wells. Cells were incubated for another 24 h, morphology was evaluated and viability determined by fluorescence microscopy through Propidium Iodide (PI) and Hoechst 33342 staining, as previously described[38].

Neuroprotection in 3D co-cultures of neurons and astrocytes

Aggregates were collected between days 38 and 50, distributed in 12- or 96-well plates and cultured in DMEM, 5% FBS, 1% P/S. Human bioavailable (poly)phenol metabolites (5 μM) were added to the cultures and 24 h after *tert*-buthyl hydroperoxide (*t*-BHP; Sigma-Aldrich) oxidative lesion was induced, for a cell viability reduction of approximately 50%, in 48 h[41]. Cell viability was accessed before exposure to test compounds, before *t*-BHP lesion induction and 48 h after lesion induction by PrestoBlue® Cell Viability Reagent (Thermo Fisher Scientific), accordingly to manufacturer's instructions. Idebenone (provided by Grupo Tecnimede) was used as positive control. Final cell viability was calculated as a percentage of cell viability before lesion induction.

Attenuation of neuroinflammation

N9 microglial cells were plated into 6-well plates (5 x 10^5 cells mL^{-1}) and then pre-incubated with 5 μM of each (poly)phenol metabolite. After 6 h, medium was discarded and cells were washed once with phosphate buffer saline (PBS) prior to addition of fresh medium with 300 ng/mL lipopolysaccharide from Escherichia coli 055:B5 (LPS, Sigma–Aldrich). Pro-inflammatory mediators release to medium were quantified as described:

NO release

The NO release to media was quantified according to[73] by Griess Reagent (Sigma–Aldrich) according to manufacturer's instructions.

TNF-α quantification by ELISA

Cell supernatants were harvested after 24 h and stored at -80 °C until analysis. Murine TNF-α release was assayed by sandwich ELISA according to the manufacturer's instructions (PeproTech®; Princeton Business Park, Rocky Hill NJ, United States)[74]. All the reagents and plates used were provided in the kit. The plate was incubated at room temperature in a Synergy HT microplate reader (Biotek®, Winooski, USA) for 35 min, with 5-min intervals Abs_{405} readings.

CD40 and superoxide ($O_2^{\bullet-}$) quantification by flow cytometry

Culture media was discarded and PBS was added to detach N9 adherent cells, which were then incubated with mouse anti-FcγR (same as CD-16/32, from E-

Biosciences) in FACS buffer (PBS containing 2% FBS and 0.01% NaN_3) for 30 minutes at 4 °C before staining. Cells were spun down at 1000 *g*, washed once with FACS buffer and stained with 5 µg/mL mouse anti-CD40 - FITC (clone 3/23, from BD Biosciences®); and with 5 µg/mL DHE probe (Dihydroethidium, Invitrogen™, Carlsbad, CA, USA) as superoxide indicator[75]. Events were acquired using CUBE 6 cytometer, from Partec®. Post-acquisition analysis was done with the software FSC express 4 flow research edition®.

Western blot

NT2 aggregates and N9 protein samples western blot analysis was performed[41]. Aggregates were lysed with TX-100 lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, pH 7.4) and N9 protein extraction was performed with RIPA buffer. Primary antibodies were incubated overnight at RT, followed by secondary antibodies (horseradish peroxidase-conjugated, ECL anti-mouse IgG, anti-rat IgG or anti-rabbit IgG; Pierce, Millipore and GE Healthcare), incubated for 2h at room temperature. Anti-GAPDH antibody (Thermo Scientific) was used as loading control. Primary antibodies used for protein detection were: anti-βIII-Tubulin (Millipore), anti-GFAP (DAKO), and MitoProfile Total OXPHOS WB primary antibody cocktail (Abcam), anti-phospho-NF-κB p65 (ser536) antibody (Cell Signalling), anti-NF-κB p65 (C-20) (Santa Cruz Biotechnology), and anti-IκB-α (C-21) antibody (Santa Cruz Biotechnology). Membranes were developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and visualized using a ChemiDocTM XRS+ System (BioRad).

Real-time quantitative PCR

Real-time quantitative PCR analysis (qRT-PCR) was performed as described in[76]. Briefly, total RNA was extracted with High Pure RNA Isolation kit (Roche) and reverse transcription performed with Transcriptor High Fidelity cDNA Synthesis kit (Roche). qRT-PCR analysis was performed in a LightCycler 480 Multiwell Plate 96 (Roche), using the Light-Cycler 480 SYBR Green I Master Kit (Roche). cDNA was diluted 1:2 and each sample was performed in triplicates. The list of used primers and its sequence is

presented in **Supplementary Table S5**. Cycles threshold (Ct's) and melting curves were determined using LightCycler 480 software, version 1.5 (Roche) and results were processed using the $2^{-\Delta\Delta Ct}$ method for relative gene expression analysis[76, 77]. Changes in gene expression were normalized using the house-keeping gene *RPL22* (coding for ribosomal protein L22) as internal control.

Statistical analysis

The results reported in this work are the averages of at least three independent experiments and are represented as the means ± SD. Differences amongst treatments were detected by analysis of variance with the Tukey HSD (honest significant difference) multiple comparison test (a = 0.05) using SigmaStat 3.10 (Systat) software.

Ethics statement

The research presented involves primary cultures of both cerebellar granule cells and microglial cells fundamental to the validation and understanding of phenolic metabolites in cell biology. All the procedures were performed in accordance with the guidelines and regulations under the DGAV approved license (0421/000/000/2013) by researchers accredited by Federation of European Laboratory Animal Science Associations (FELASA)/Direcção Geral de Alimentação e Veterinária (DGAV). Animals used for primary cultures extraction purpose were provided by "Instituto Gulbenkian de Ciência" Animal Facility in compliance with the Portuguese and European laws (Directive 2010/63/EU on the protection of animals used for scientific purposes), under regulation of the Portuguese official Veterinary Directorate (DGAV), which complies with the European Directive and follows the FELASA guidelines and recommendations concerning laboratory animal welfare.

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Contributions

IF designed the research, performed the laboratory experiments, performed MS and general data analysis and interpretation, and prepared the manuscript; GG performed the laboratory experiments with microglia and performed data analysis; RCP contributed to the MS data interpretation in BBB transport assays; APT performed the laboratory experiments and contributed to the NT2 3D culture assays; IC performed the laboratory experiments with mouse cerebellar granule cells; AFA designed and performed *in silico* predictions and interpretation; LT contributed to mouse cerebellar granule cells data interpretation; TFP contributed to the design of microglial cells experiments; PP contributed to microglial cells data interpretation; MRV performed *in silico* predictions data analysis and interpretation, and critically revised the manuscript; AF contributed to the NT2 3D cell culture experiments; GMcD performed MS data interpretation and

critically revised the manuscript; DS performed MS data interpretation and critically revised the manuscript; KK provided HBMEC line and contributed to the BBB transport assays; IP contributed to the BBB transport assays; DB contributed to the BBB transport assays; MAB designed the research, contributed to data analysis and interpretation and critically revised the manuscript; CB designed the research, contributed to data analysis and interpretation and critically revised the manuscript; CNS designed the research, contributed to data analysis and interpretation and critically revised the manuscript.

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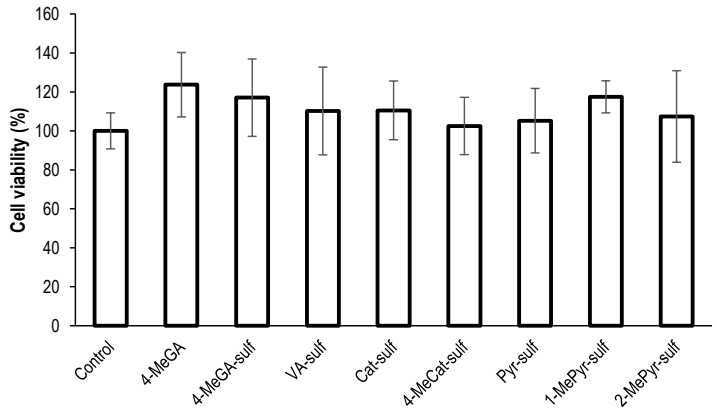
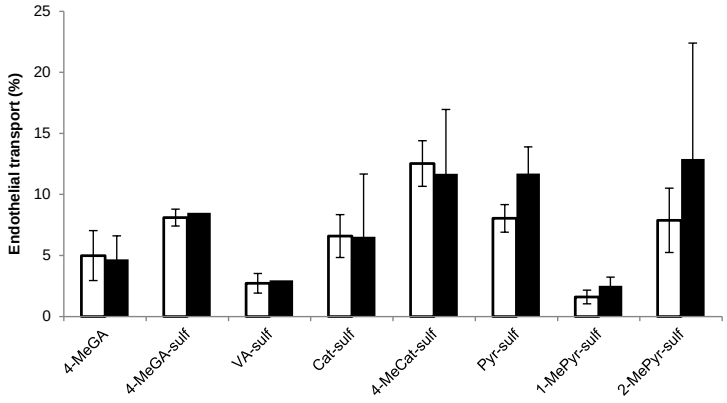
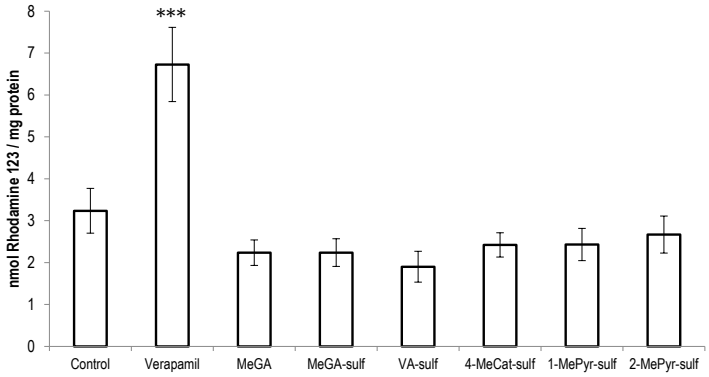
Supplementary material



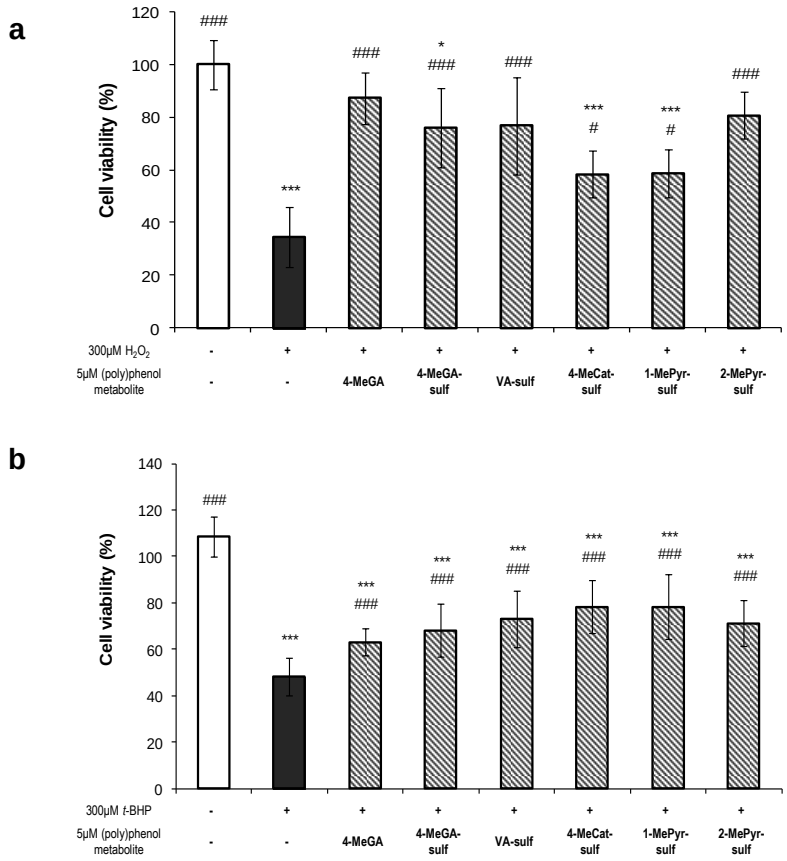
Supplementary Figure S1. Toxicity of human bioavailable (poly)phenol metabolites in HBMEC line. Cells were pre-incubated with 5µM of each bioavailable polyphenol metabolite for 24h. Cell viability was assessed and is presented as percentage relatively to control. All values are means ± SD, n=3.



Supplementary Figure S2. Blood-brain barrier transport of human bioavailable (poly)phenol metabolites. Endothelial transport of 5M (white) or (black) of each human bioavailable polyphenol metabolite after 2h of incubation. Endothelial transport was evaluated by LC-Orbitrap MS and is presented as percentage (%) determined by the ratio of the lower compartment concentration and the sum of the upper and lower compartments concentrations.

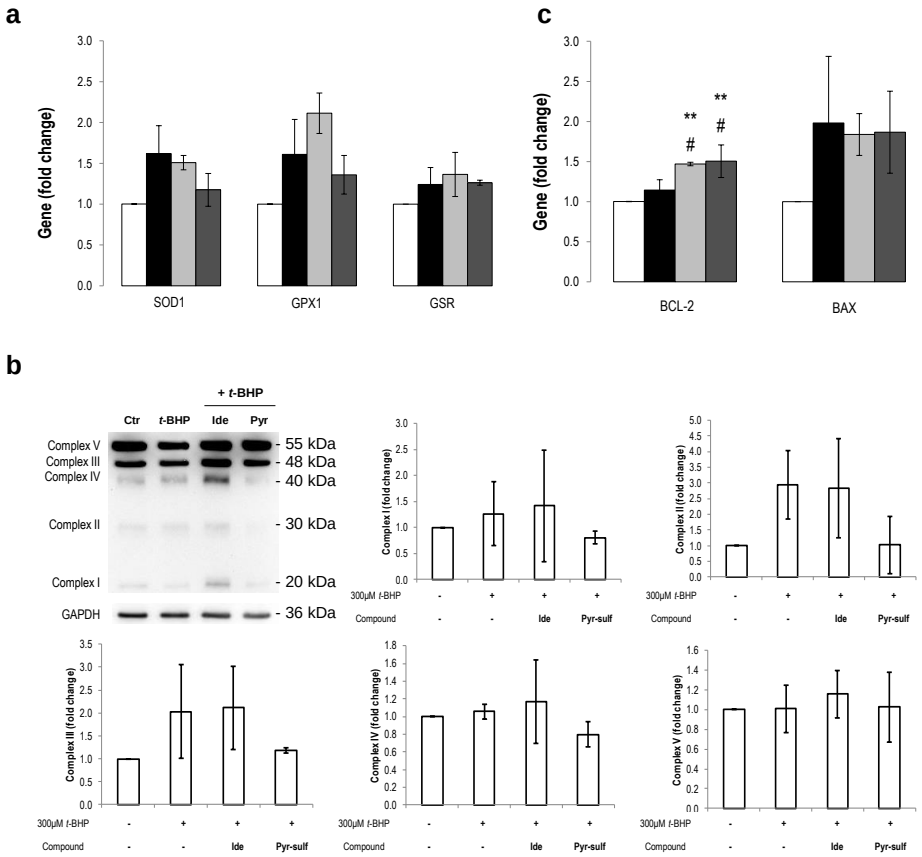


Supplementary Figure S3. P-glycoprotein substrate accumulation for (poly)phenol metabolites tested. Intracellular accumulation of P-gp substrate, Rhodamine 123 was evaluated by fluorescence after pre-incubation of cells with each of the bioavailable (poly)phenol metabolites compared with P-gp inhibitor, verapamil. Results are presented normalized for protein content. All values are means ± SD, n=3.

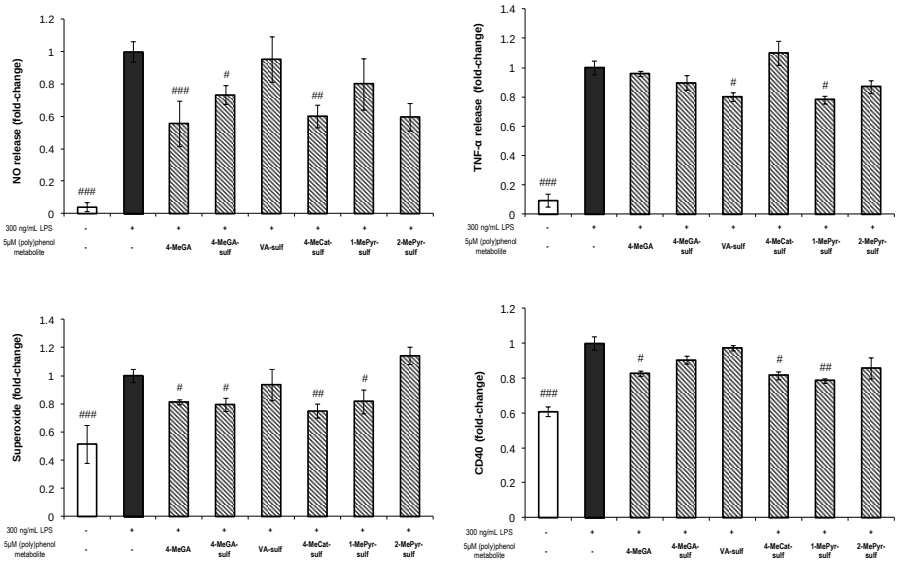


Supplementary Figure S4. Cytoprotective potential of human bioavailable (poly)phenol metabolites in different cell systems: (**a**) HBMEC line submitted to oxidative stress (300μM H_2O_2); (**b**) 3D aggregates exposed to oxidative injury (300μM *t*-BHP). Cells were pre-incubated with 5μM of each bioavailable polyphenol metabolite for 24h and then injured with the respective insult. Cell viability was assessed and is presented as percentage relatively to control. Statistical differences are denoted as *** p<0.001, ** p<0.01

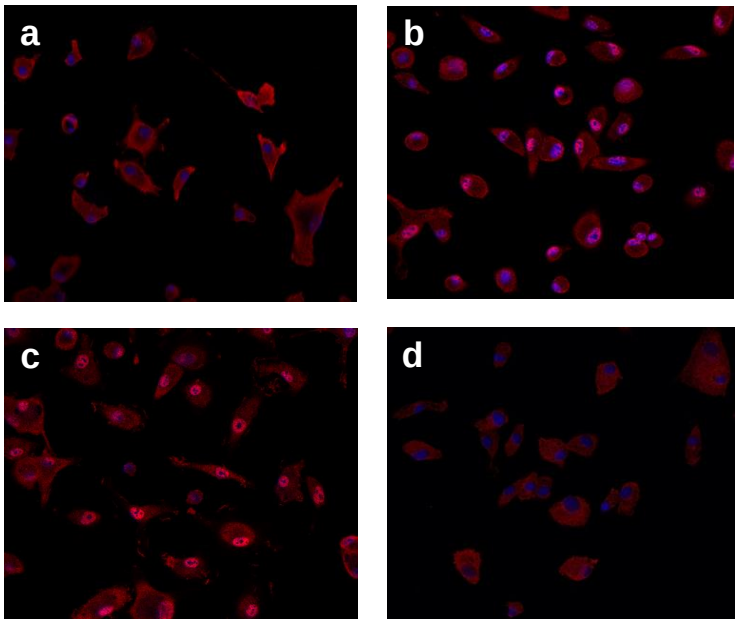
and * p<0.05 relatively to control and as ###p<0.001, ##p<0.01 and #<0.05 relatively to each insult (H_2O_2 or *t*-BHP). All values are means ± SD, n=3.



Supplementary Figure S5. Alterations in oxidative stress, mitochondrial complexes and apoptosis in 3D aggregates. (**a**) Levels of gene expression of *SOD1*, *GPX1* and *GSR*, or (**c**) *BAX* and *BCL-2* in control 3D aggregates (white), 3D aggregates injured with *t*-BHP (black), 3D aggregates pre-incubated with 200nM Idebenone (Ide) and injured with *t*-BHP (light grey) and 3D aggregates pre-incubated with 5µM Pyr-sulf and injured with *t*-BHP (dark grey). Changes in gene expression were normalized using the housekeeping gene RPL22 (coding for ribosomal protein L22) as internal control. (**b**) Protein levels of mitochondrial subunits of the respiratory chain in 3D aggregates: representative western blot and corresponding fold changes in protein levels normalized to GAPDH. Statistical differences are denoted as **p<0.01 and *p<0.05 relatively to control and as #p<0.05 relatively to *t*-BHP. All values are mean (fold change relative to control) ± SD of three independent experiments.



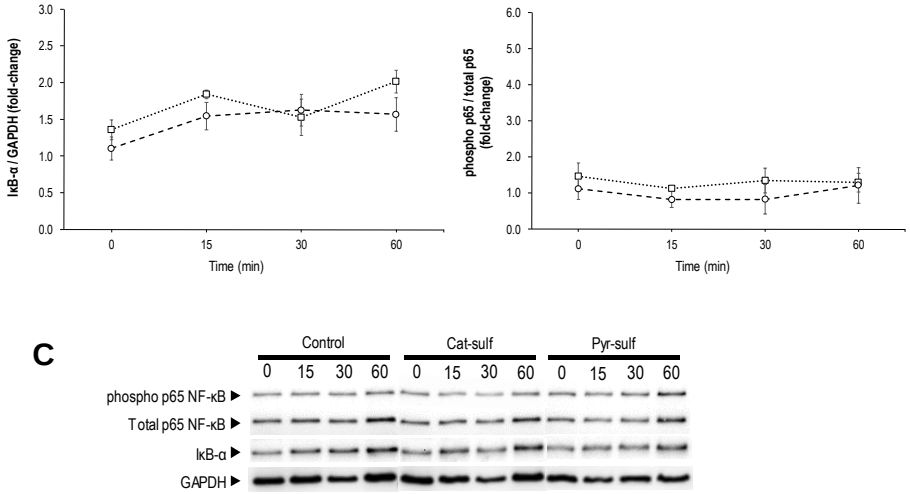
Supplementary Figure S6. Human bioavailable polyphenol metabolites effects on neuroinflammation. Pro-inflammatory markers were evaluated, namely (**a)** nitric oxide, detected by Griess reaction, (**b)** TNF-α release, detected by quantitative ELISA, intracellular superoxide production (**c**) and CD40 (**d**) quantified by flow cytometry in N9 murine microglial cells. Cells were pre-incubated for 6h with each of the bioavailable (poly)phenol metabolites and then challenged with 300ng/mL of LPS. Statistical differences are denoted as ###p<0.001, ##p<0.01 and #<0.05 relatively to lesion (LPS). All values are means ± SD, n=3.



Supplementary Figure S7. Microglial NF-κB p65 translocation into the nucleus after 60 minutes of LPS stimulation. Immunofluorescence microscopy images of primary cultures of rat microglia*: (**a**) control cells, (**b**) microglia stimulated with LPS only, and (**c**) microglia pre-treated with Cat-sulf or (**d**) Pyr-sulf for 6 h before LPS-stimulation. NF-κB (red); Nuclei (cyan) stained with DAPI. Scale bar: 30 µm.

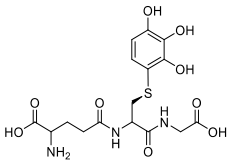
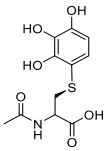
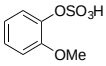
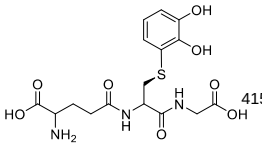
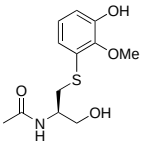
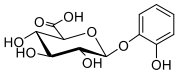
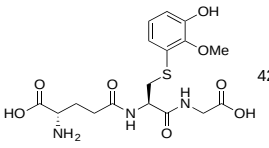
*** Establishment of rat primary cell cultures of microglia**

Briefly, new born Wistar rats were dissected and the heads removed. Cortices were placed into petri dishes and meninges removed. Brains were mechanically disrupted and the suspension centrifuged. Cells were cultured in high-glucose DMEM with Glutamax and supplemented with 10% FBS, 5 µg/mL insulin, 2.0 mg/mL L-glucose and 1% Pen/Strep for 14–21 days. Granulocyte-macrophage colony-stimulating factor was added at the beginning of the culture and subsequently every 3 days at a final concentration of 0.25 ng/mL. At day 14, confluent mixed glial cell cultures were shaken for 2 h at 200 rpm. Microglial cells were obtained from the supernatant after filtering through 100-µm cell strainers. Cells were re-suspended in DMEM M2279 with Glutamax supplemented with 10% FBS, 50 µM β-mercaptoethanol and 1% Pen/Strep. Cells were plated in 24 well plates for 1 h. Adherent cells were washed twice with PBS to remove cell debris and astrocytes.

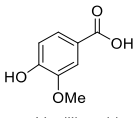
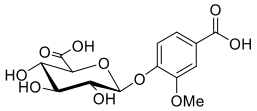
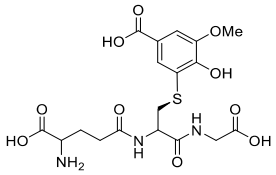


Supplementary Figure S8. Microglial NF-κB p65 phosphorylation ratio and IκBα protein levels for cells treated only with Cat-sulf and Pyr-sulf. (**a**) IkBα protein levels along time. (**b**) NF-κB activation profile along time looking at NF-κB p65 phosphorylation (ser536) ratio. Cells were pre-treated either with Pyr-sulf (white squares, dotted line) or Cat-sulf (white circles, dashed line). All values are means ± SD, n=3. (**c**) Representative western blot for control cells and cells treated only with (poly)phenol metabolites as indicated.

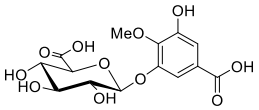
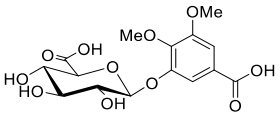
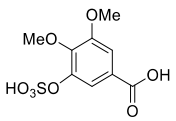
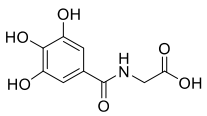
Supplementary Table S1. Putative new cellular metabolites arising from human bioavailable (poly)phenol metabolites in HBMEC, namely from Pyr-sulf and Cat-sulf. Using in-house database of predicted human bioavailable (poly)phenol metabolites and taking advantage of Orbitrap-MS data collected, search for novel metabolites was performed using Xcalibur QualBrowser software. Putative ID of predicted metabolites was determined by exact mass, according to their appropriate *m/z* value in both upper and lower compartment samples for each parent compound.

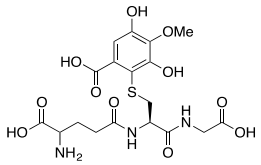
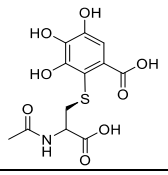
Parent compound	Metabolite nomenclature	Structure	Exact mass	m/z	Apical	Basolateral	Other parents compounds
Pyr-sulf	*Glutathionyl-pyrogallol*		431,0998	430,4	+	+	1-MePyr-sulf 2-MePyr-sulf
Pyr-sulf	*Acetylcysteine-pyrogallol*		287,0464	286,3	+	+	1-MePyr-sulf 2-MePyr-sulf
Pyr-sulf	*2-O-methylcatechol-O-sulfate*		204,0092	203,2	+	+	1-MePyr-sulf 2-MePyr-sulf
Pyr-sulf	*Glutathionyl-catechol*		415,1049	414,4	+	-	-
Pyr-sulf Cat-sulf	*Acetylcysteine-2-O-methylcatechol*		301,0620	300,1	+	+	4-MeCat-sulf 1-MePyr-sulf 2-MePyr-sulf
Cat-sulf	*2-O-methylcatechol*		124,0524	123,1	+	+	-
Cat-sulf	*Catechol-1-O-glucuronide*		286,0689	285,2	+	+	4-MeCat-sulf
Cat-sulf	*Glutathionyl-2-O-methylcatechol*		429,1206	428,4	+	+	4-MeCat-sulf

Supplementary Table S2. Putative new cellular metabolites arising from human bioavailable (poly)phenol metabolites in HBMEC, namely from VA-sulf. Using in-house database of predicted human bioavailable (poly)phenol metabolites and taking advantage of Orbitrap-MS data collected, search for novel metabolites was performed using Xcalibur QualBrowser software. Putative ID of predicted metabolites was determined by exact mass, according to their appropriate m/z value in both upper and lower compartments samples for each parent compound.

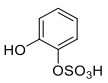
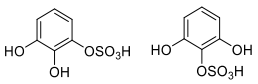
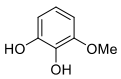
Parent compounds	Metabolite nomenclature	Structure	Exact mass	m/z	Apical	Basolateral
VA-sulf	*Vanillic acid*		168,0423	167,2	+	-
VA-sulf	*Vanillic-4-O-glucoronide*		344,0743	343,3	+	+
VA-sulf	*Glutathionyl-vanillic acid*		473,1104	472,4	+	+

Supplementary Table S3. Putative new cellular metabolites arising from human bioavailable (poly)phenol metabolites in HBMEC, namely from 4-MeGA and 4-MeGA-sulf. Using in-house database of predicted human bioavailable (poly)phenol metabolites and taking advantage of Orbitrap-MS data collected, search for novel metabolites was performed using Xcalibur QualBrowser software. Putative ID of predicted metabolites was determined by exact mass, according to their appropriate m/z value in both upper and lower compartments samples for each parent compound.

Parent compounds	Metabolite nomenclature	Structure	Exact mass	m/z	Apical	Basolateral
4-MeGA 4-MeGA-sulf	*4-O-methylgallic acid-3-O-glucuronide*		360,0693	359,3	+	+
4-MeGA 4-MeGA-sulf	*4,5-O-dimethylgallic acid-3-O-glucuronide*		374,0849	373,3	+	+
4-MeGA 4-MeGA-sulf	*4,5-O-dimethylgallic acid-3-O-sulfate*		278,0096	277,2	+	+
4-MeGA-sulf	*Galloylglycine*		227,0430	227,2	+	+

4-MeGA 4-MeGA-sulf	*Glutathionyl-4-O-methylgallic acid*		489,1053	488,4	+	+
4-MeGA	*Acetylcysteinyl-gallic acid*		331,0362	330,3	+	+

Supplementary Table S4. Putative new cellular metabolites arising from human bioavailable (poly)phenol metabolites in HBMEC, namely from 4-MeCat-sulf, 1-MePyr-sulf and 2-MePyr-sulf. Using in-house database of predicted human bioavailable (poly)phenol metabolites and taking advantage of Orbitrap-MS data collected, search for novel metabolites was performed using Xcalibur QualBrowser software. Putative ID of predicted metabolites was determined by exact mass, according to their appropriate m/z value in both upper and lower compartments samples for each parent compound.

Parent compound	Metabolite nomenclature	Structure	Exact mass	m/z	Apical	Basolateral
4-MeCat-sulf	*Catechol-1-O-sulfate*		189,9936	189,2	+	+
1-MePyr-sulf 2-MePyr-sulf	*Pyrogallol-O-sulfate*		205,9885	205,2	+	-
1-MePyr-sulf 2-MePyr-sulf	*1-O-methylpyrogallol*		140,1366	139,1	+	-

Supplementary Table S5. List of primers used in qRT-PCR analysis.

Gene	Cell type/Function	Primers forward (top) and reverse (bottom)
RPL22	Ribosomal protein L22 (housekeeping gene)	CACGAAGGAGGAGTGACTGG TGTGGCACACCACTGACATT
SOD1	Superoxide dismutase 1	AGGCCCCTTAACTCATCT CTACAGGTACTTTAAAGCAACTCT
GPX1	Glutathione peroxidase 1	TTTGGGCATCAGGAGAACGC AGCATGAAGTTGGGCTCGAA
GSR	Glutathione reductase	CGTGGAGGTGCTGAAGTTCTC TCATGGTCATGACTGGTAGCC
BCL-2	B-cell lymphoma 2	ATCGCCCTGTGGATGACTGAG CAGCCAGGAGAAATCAAACAGAGG
BAX	BCL-2 associated X protein	TGGAGCTGCAGAGGATGATTG GAAGTTGCCGTCAGAAAACATG

Chapter 4

Bioavailable polyphenol metabolites are neuroprotective in Parkinson's disease

This chapter is based on the following manuscript:

Circulating (poly)phenol metabolites: neuroprotection in a 3D cell model of Parkinson's disease

Figueira I, Terrasso AP, Godinho-Pereira J, Leist M, Brito C, Santos CN (under preparation)

This chapter contains data in which the author of this dissertation executed the majority of the experiments. Supplementary Fig S1b and supplementary Fig S2a and S2c were performed by A. P. Terrasso.

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Abstract

Parkinson's disease (PD) is one of most common age-related neurodegenerative disorders, still without cure. The prevention and treatment of neurodegeneration, characterized by a multifactorial phenotype, require novel therapeutic strategies. Epidemiological and clinical studies have proven that diets rich in (poly)phenols are able to reduce the incidence and prevalence of such disorders, exhibiting remarkable multipotent ability to modulate several pathways. Thus, (poly)phenols, proven to act as pleiotropic agents, can constitute a promising alternative to prevent PD onset and progression. Our goal was to investigate the potential protective effects against PD of bioavailable metabolites derived from dietary (poly)phenols, identified in an human intervention study. A 3D human brain cell model was generated by differentiation of Lund human mesencephalic neural progenitor cells (LUHMES) in an agitation-based culture system. Differentiated neurospheroids, enriched in dopaminergic neurons and presenting synaptic activity were obtained. (Poly)phenol metabolites differentially modulated central signaling pathways, such as Nrf2 and p53, in LUHMES neurospheroids. The neuroprotective effect of these bioavailable (poly)phenol metabolites was confirmed in a PD cell model, based in 1-methyl-4-phenylpyridinium (MPP+) lesion over LUHMES neurospheroids. These (poly)phenol metabolites were proven to act through hormesis mechanism and to modulate key genes involved in apoptosis and Nrf2 signaling, emerging as potential new candidates to prevent PD disease onset and progression.

Introduction

The increase in life expectancy of the developed countries has led to a higher incidence of chronic disorders like neurodegenerative disorders. Parkinson's disease (PD) is the second most common neurodegenerative disorder and the first motor debilitating degenerative disease[1, 2]. PD is a chronic, incurable disorder, which affects 1–3% of the elderly population worldwide and the great majority of PD cases are sporadic with no apparent genetic association [3]. Changes found in the brain of PD patients comprise the loss of dopaminergic neurons in the *substantia nigra pars compacta* and the presence of protein aggregates containing α-synuclein[4]. PD symptoms result from a decrease in dopamine levels which might be caused by a disturbance of dopamine exchange system functioning (synthesis, transport, synaptic release, and reception). Proteins associated with dopamine synthesis and retention include tyrosine hydroxylase (TH), dopamine transporter (DAT), monoamine vesicular transporter (VMAT2), and aromatic amino acid decarboxylase (AADC)[5]. Among several factors, the regulation of TH activity and gene transcription play a large role in PD pathogenesis[5].

Dopaminergic neurons in PD display defects in mitochondrial function, increased oxidative stress and neuroinflammation[6]. Through the past years, it has been shown that oxidative stress is central to the pathology of this disease[7]. Moreover, the *substantia nigra* of PD patients shows increased levels of oxidized lipids, proteins and DNA, together with a decrease in reduced glutathione (GSH) levels[8]. In PD, a main source of free radicals stems from alterations in complex I of the mitochondrial respiratory chain, which compromises proper mitochondrial functioning and ATP production[9]. Increased neuroinflammation in PD is also a consequence of increased reactive oxygen species (ROS) and leads to microglia secretion of cytokines, causing the activation of the transcription factor NF-κB[10]. Such activation enhances the sensitivity of dopaminergic neurons to oxidative stress and ultimate cell death.

Development of PD may also be linked with the toxic action of environmental factors. As such, enzymes involved in detoxification systems, both from phase I (*i.e.* proteins of the cytochrome P450 superfamily), or phase II [*i.e.* glutathione-S-transferases (GSTT1, GSTM1, GSTP1) and N-acetyl transferase (NAT2)] metabolism, can play an important

role in the pathogenesis of the disease[5]. As such, the multi-factorial nature of PD contributes to the process of nigral cell death, of which mitochondrial dysfunction and increased production of ROS are the most important mechanisms. The multiple primary causes of PD may be ultimately linked to a final common signal-transduction pathway leading to programmed cell death of the dopaminergic neurons[11].

The prevention and treatment of PD, characterized by a mechanistic complexity, need novel therapeutic strategies targeted to multiple genes and proteins. In fact, PD usually starts long before its manifestations appear. By taking advantage of diets that may be able to prevent disease onset and/or progression is important [12]. Nowadays, attention is being given to (poly)phenols as strong attenuators of oxidative stress and inflammation, key processes in PD progression, presenting fewer side effects than most drugs available. (Poly)phenols exhibit a remarkable multipotent ability to modulate, for instance, oxidative stress, metal toxicity, inflammation and immune response, apoptosis, signal transduction, ion channels, among others (reviewed in [13]). Studies have showed that dietary (poly)phenols are able to reduce the incidence and prevalence of neurodegenerative disorders, with significant benefits for brain health[14-16]. Moreover, there is growing evidence of the potential against different cellular and molecular mechanisms of PD of (poly)phenols. For instance, (poly)phenols like resveratrol[17, 18], epigallocatechin gallate[19, 20], quercetin [21] and apigenin[22] have shown to be able to modulate PD associated progressive dopaminergic degeneration, mitochondrial dysfunction, apoptosis, protein aggregation and neuroinflammation. However, the mechanism of action of (poly)phenol metabolites, simpler compounds which result from human metabolism over dietary (poly)phenols, in PD is still only marginally addressed[23, 24]. Such circulating metabolites can present a promising strategy for disease prevention with potential to be explored. Simple phenolic sulfates, colonic-derived metabolites of dietary (poly)phenols were identified in biological samples of human volunteers which took a berries mixture[25, 26], cranberries[27], mango[28] or black tea[29] . Such sulfates, when tested in circulating concentrations, were shown to be significantly transported across a simplified *in vitro* model of the blood-brain barrier (BBB)[30], holding great promise as possible brain-targeted compounds. In fact, these metabolites presented strong

neuroprotective and anti-neuroinflammatory potential against common neurodegeneration hallmarks *in vitro*[30].

The purpose of this study was to investigate potential protective effects of selected human bioavailable metabolites derived from the degradation of dietary (poly)phenols[26].A 3D human brain cell model generated from LUHMES human neuronal precursor cells was used. LUHMES were differentiated into neurospheroids containing post-mitotic neurons with enrichment in dopaminergic neurons[31-33] in agitation-based culture system. For induction of a PD-like phenotype, LUHMES neurospheroids were treated with the dopaminergic neurotoxicant 1-methyl-4-phenylpyridinium (MPP^+), an inhibitor of complex I of the mitochondrial respiratory chain with high affinity to the dopamine transporter of dopaminergic neurons, leading to incomplete oxygen reduction and generation of ROS, with depletion of overall ATP levels[32]. The 3D cell model of PD was employed to assess the effects of physiological concentrations (poly)phenol metabolites. (Poly)phenol metabolites were not toxic to neurospheroids and were neuroprotective towards the dopaminergic lesion applied. Moreover, these metabolites were able to modulate genes related with central canonical pathways involved in stress response. Major modulated genes, such as *ATF4* and *BCL2,* seem to be involved in the neuroprotection observed, with relevance towards the molecular hallmarks of PD. To our knowledge, this is the first study assessing the potential of circulating (poly)phenol metabolites in a 3D cell model of PD.

Results

Circulating (poly)phenol metabolites are neuroprotective towards MPP^+

Simple phenolic sulfates, colonic-derived metabolites of dietary (poly)phenols which were identified in blood and urine samples of a human intervention study[25, 26], were shown to be significantly transported across a simplified *in vitro* model of the BBB, being neuroprotective against different neurodegeneration hallmarks[30]. As such, these phenolic sulfates may comprise a great promise as possible brain-targeted compounds but never assessed in a specific brain pathology. To evaluate their potential, we employed a PD cell model of MPP^+-induced lesion in a 3D human cell system. Differentiated LUHMES neurospheroids were obtain and presented to be enriched in post-mitotic neurons (**Supplementary Fig. S1**), as indicated by the increased mRNA and protein levels of major neuronal (*e.g.* βIII-tubulin, synaptophysin) and dopaminergic (*e.g.* TH, DAT) markers, with concomitant decrease in early neuroepithelial progenitors markers (*e.g.* Nestin). These neurospheroids were also enriched dopaminergic neurons, as confirmed by the presence of TH-positive neurons (**Supplementary Fig. S2**). MPP^+ lesion reduced cell viability in a dose-dependent manner, with an IC_{50} of 5μM. A downregulation in the dopaminergic TH gene expression was observed along lesion time, with no significant alterations in βIII-tubulin and DAT gene expression (**Supplementary Fig. S3**). Interestingly, protein levels of both βIII-tubulin and TH did not significantly decrease with MPP^+ lesion (**Supplementary Fig. S3**).

LUHMES neurospheroids were incubated for 24h with physiologic-circulating concentrations (5μM) of two human phenolic sulfates: catechol-sulfate (Cat-sulf) and pyrogallol-sulfate (Pyr-sulf)[25, 26, 30] (**Fig. 1a**) and assessed in parallel with Idebenone (Ide), a known antioxidant drug which is a short-chain analogue of coenzyme Q10[34, 35]. Ide treatment lead to a reduction in oxidative stress markers and was shown to protect against ROS-induced damage in several *in vitro* cultures, as primary cortical neurons and immortalized neural cells[34]. Ide was shown to rescue ATP levels under conditions

of impaired mitochondrial complex I[35], such as the conditions caused by MPP^+ lesion in our system.

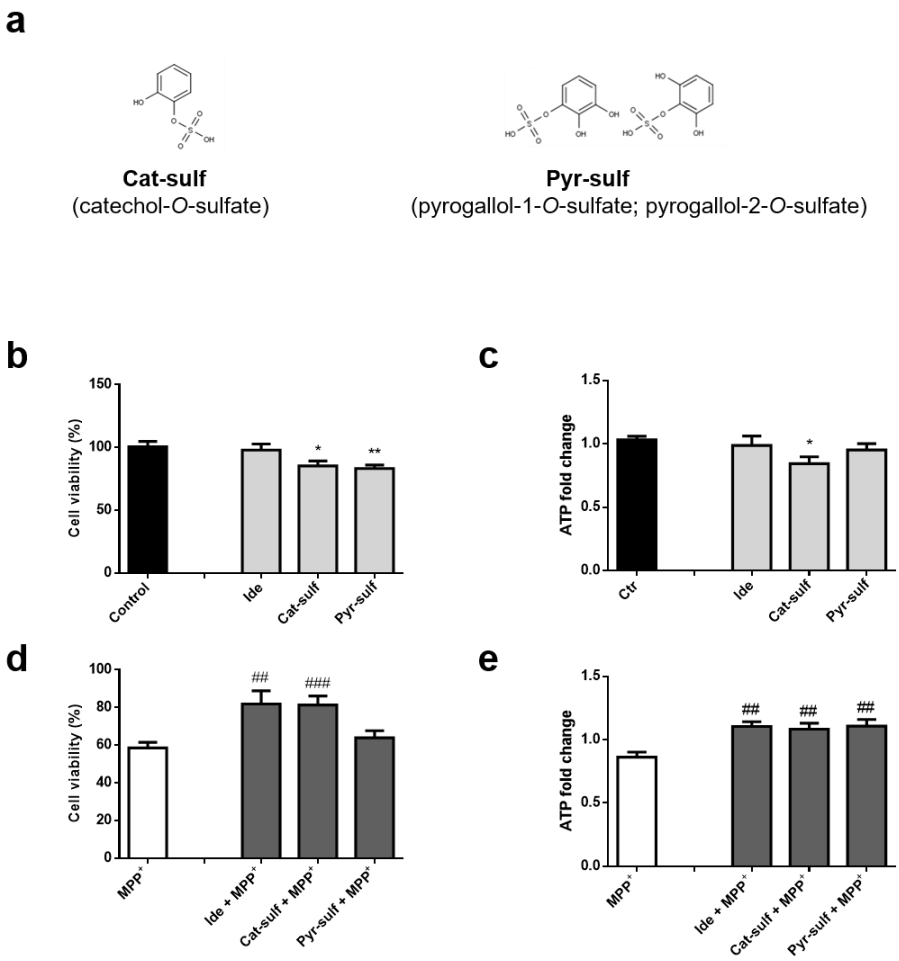

Fig. 1. Toxicity and neuroprotective potential of (poly)phenol metabolites against MPP^+ lesion in LUHMES neurospheroids. (**a**) Chemical structure representation of catechol-*O*-sulfate (Cat-sulf) and pyrogallol-O-sulfate (Pyr-sulf, a mixture of two isomers). Toxicity of dietary (poly)phenol metabolites (Cat-sulf, Pyr-sulf) and Idebenone (Ide) in LUHMES neurospheroids after 24h of incubation in terms of (**b**) cell viability and (**c**) ATP levels. Neuroprotection evidenced in terms of (**d**) cell viability or (**e**) ATP fold change. LUHMES neurospheroids were pre-incubated with different dietary (poly)phenol metabolites (5μM Cat-sulf, 5μM Pyr-sulf) or with 200nM Ide for 24h and then treated or not with 5μM of MPP^+. Cell viability was assessed by Presto blue assay after 24h of injury. ATP fold change was assessed by CellTiter Glo Assay after 48h of injury and is expressed relatively to control condition (without MPP^+). Statistical differences are denoted as **p<0.01 and *p<0.05 relative to control and denoted as ###p<0.001 and ##p<0.01 relative to MPP^+ insult obtained by one-way ANOVA analysis with Tukey's post multiple comparison test. Data are mean ± SEM of three independent cultures.

By opposition to Ide, both Cat-sulf and Pyr-sulf presented a slight toxicity in LUHMES neurospheroids in terms of cell viability, reducing it to just about 85% (**Fig. 1b**). Moreover, Cat-sulf also induced a small but significant decrease in ATP production (**Fig. 1c**). Despite these effects, Cat-sulf and Pyr-sulf were shown to be protective over an MPP^+ lesion, although in different extents. Only the pre-incubation with Ide or Cat-sulf was able to maintain cell viability higher than in MPP^+ lesion and at the same extent (**Fig. 1d**). Moreover, both metabolites pre-incubation, Cat-sulf and Pyr-sulf, and Ide pre-incubation as well were capable to increase ATP intracellular levels (**Fig. 1e**).

Circulating (poly)phenol metabolites modulate central signaling pathways

The effects of both Cat-sulf and Pyr-sulf at the circulating concentrations in LUHMES neurospheroids (**Fig. 1b** and **1c**) suggest that they can be promoting a pre-conditioning hormetic effect: low doses of these phenolic sulfates can be triggering a cellular response that help them to cope later with the MPP^+ lesion (as observed, **Fig. 1d** and **1e**). To evaluate the mechanism and signaling pathways underlying this protective response, a custom PCR array was performed to evaluate key players in the top signal transduction pathways. Of the total 93 target genes assessed (**Table S2**), 66 were amplified. Both Cat-sulf and Pyr-sulf treatment induced changes in gene transcription, with fold changes ranging from -4.63 to 13.7, for Cat-sulf, and ranging from -3.12 to 7.08, for Pyr-sulf comparatively to untreated (control) neurospheroids.

Taking advantage of functional enrichment analysis of Ingenuity Pathway Analysis (IPA) software, major Canonical Pathways and Upstream Regulators were identified as significantly modulated by Cat-sulf and Pyr-sulf in LUHMES neurospheroids (**Fig. 2-4**). Enrichment analysis delivered the significant over-represented pathways across the list of differentially expressed genes (**Fig. 2a**). In this analysis, nuclear factor erythroid 2-related factor 2 (*Nrf2)-mediated Oxidative Stress Response* was identified as the top canonical pathway, with the highest -log(*p*-value) and high z-score, and comprising mainly upregulated target genes under Nrf2 regulation: the Antioxidant Responsive Elements (ARE). Nrf-2 signaling pathway was predicted to be more activated (higher activation z-score) by Pyr-sulf than by Cat-sulf (**Fig. 2a** and **2b**). Genes such as *RAF1* (c-Raf), *MAP3K1* (MEKK) and *AKT1* (AKT), upstream of Nrf2/Keap1 complex

disassembly, as well as genes under ARE promoter such as chaperones and stress response proteins (*HERPUD1*, *HSP90AA*, *HSP90B1*), phase I and II metabolizing enzymes (*NQO1*, *GCLC*, *GCLM*), and antioxidant proteins (*SQSTM1*, *ATF4*, *SOD*, *HMOX1*, *GSR*, *TXNRD1*, *FTH1*) were significantly modulated in the presence of Cat-sulf and Pyr-sulf (**Fig. 2b**).

Peroxisome Proliferator-Activated Receptors *(PPAR) signaling* was the canonical pathway predicted to be more inhibited (lowest activation z-score) by both Cat-sulf and Pyr-sulf (**Fig. 2a**). PPAR act as ligand-activated transcription factors leading to the transcription of target genes involved in the catabolism of fatty acids [36].

A stronger effect of Pyr-sulf towards *NF-κB signaling* canonical pathway was also observed in LUHMES neurospheroids (**Fig. 2a**), as well as in *JAK/STAT signaling* (**Fig. 2a** and **2c**). In fact, Cat-sulf and Pyr-sulf presented opposite effects in this canonical pathway: Cat-sulf seems to lead to the inhibition of JAK/STAT cascade, while Pyr-sulf leads to a slight activation of this pathway (**Fig. 2a** and **2c**). For instance, Cat-sulf leads to a strong down-regulation of *AKT1* and *BCL2L1* genes, with the up-regulation of *SOCS3* gene (a JAK2 kinase inhibitor), while Pyr-sulf leads to an up-regulation of genes coding for STAT complex, leading to an overall predicted activation of the pathway (**Fig. 2c**).

By opposition, Cat-sulf lead to a higher activation of *p53 signaling* canonical pathway than Pyr-sulf (**Fig. 2a**). In fact, TP53 emerged as a strong upstream regulator predicted to be activated by Cat-sulf (**Fig. 3a**) and also, in a lesser extent, by Pyr-sulf (**Fig. 3b**). Downstream of TP53 genes like *ATF4*, *BAX*, *BCL2L1* or *GSR* presented to be significantly modulated in both datasets (**Fig. 3a** and **3b**).

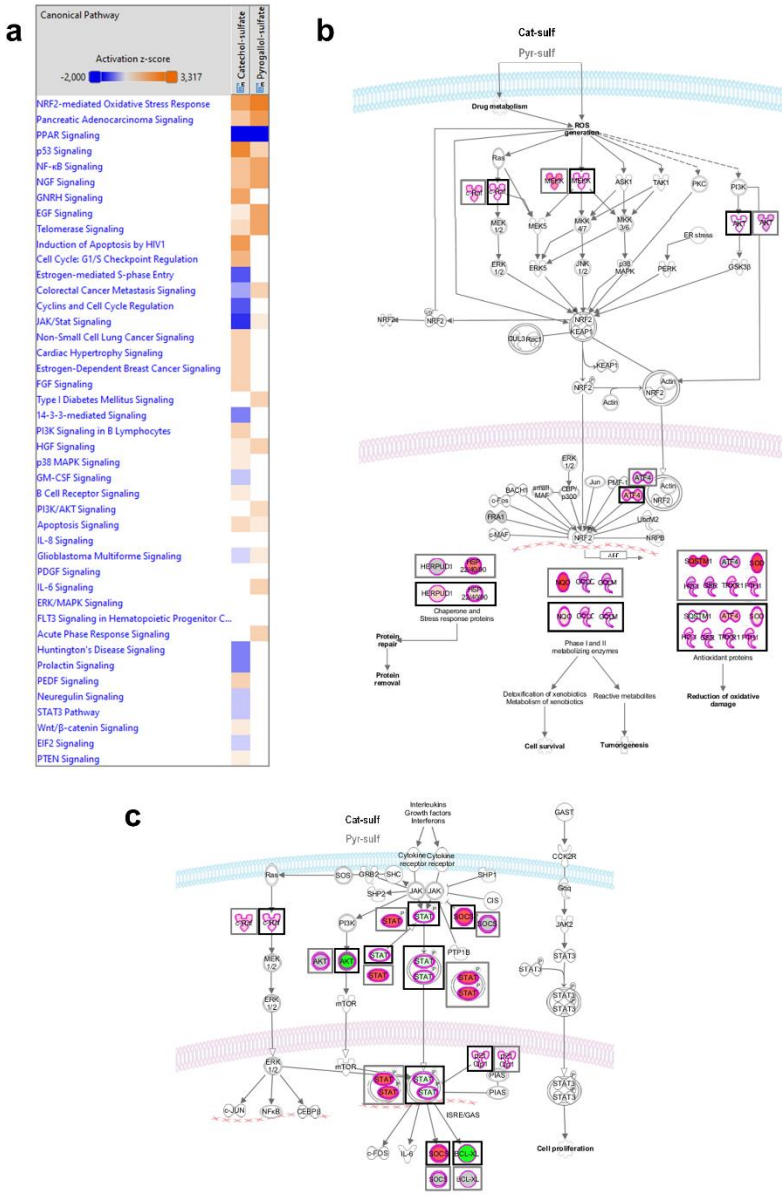

Fig. 2. Circulating (poly)phenol metabolites effects in canonical pathways in LUHMES neurospheroids. (**a**) Heat map of the canonical pathways significantly modulated by (poly)phenol metabolites in neurospheroids obtained with Ingenuity® Pathway Analysis (IPA). Pathways are ordered by activation z-score (predicted activation, higher z-score – orange; predicted inhibition, lower z-score – blue). (**b**)*Nrf2-mediated oxidative stress response* pathway obtained with IPA from the analysis of dataset modulated by Cat-sulf and by Pyr-sulf. (**c**) *JAK/STAT signalling* pathway obtained with IPA from the analysis of dataset modulated by Cat-sulf and by Pyr-sulf. Genes whose expression was significantly modulated by Cat-sulf are surrounded in black and modulated by Pyr-sulf are surrounded in grey, together with other genes involved in the cascade (but not significantly modulated, not colored). Up-regulated genes are presented in red, down-regulated genes are presented in green, where a higher intensity in the color reflects a higher degree of gene modulation.

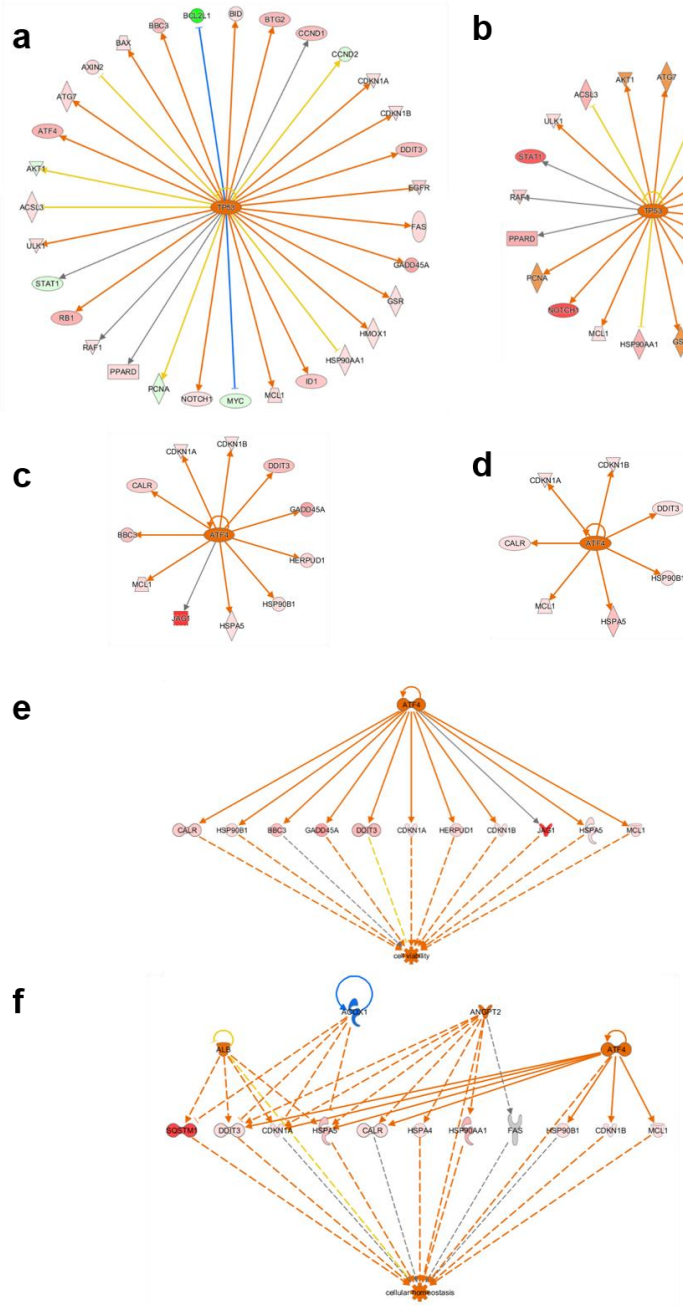

Fig. 3. Circulating (poly)phenol metabolites effects in upstream regulators in LUHMES neurospheroids. Identification of TP53 as an upstream regulator of transcriptional regulation by (**a**) Cat-sulf and by (**b**) Pyr-sulf. Identification of ATF4 as an upstream regulator of transcriptional regulation by (**c**) Cat-sulf and by (**d**) Pyr-sulf. Bioinformatic analysis with IPA software predicted the activation of both TP53 and ATF4 (orange) as a regulators of genes that are upregulated (depicted in red) and downregulated (depicted in green). Regulator effects of ATF4 towards cellular functions for (**e**) Cat-sulf and (**f**) Pyr-sulf. Bioinformatic analysis with IPA software predicted the activation of ATF4 (orange) as a regulator of genes that are upregulated (depicted in red) and downregulated (depicted in green). Arrows represent predicted relationships: orange

– leads to activation; blue – leads to inhibition; yellow – findings inconsistent with state of downstream molecule; grey – effect not predicted.

Another upstream regulator identified in both datasets was the transcription factor ATF4 (**Fig. 3c** and **3d**). Regulator Effects tool of IPA software integrated results from Upstream Regulator and Downstream Effects tools, creating hypotheses to explain the mechanisms triggered both by Cat-sulf (**Fig. 3e**) and Pyr-sulf (**Fig. 3f**). For Cat-sulf, the predicted activation of ATF4 lead to the upregulation of genes involved in heat shock response (*HERPUD1*, *HSPA5*), in endoplasmic reticulum stress and unfolded protein response (*CALR, DDIT3, HSP90B1*) and in cell cycle control (*CDKN1A, CDKN1B, GADD45A*), leading to an overall increase in cell viability (**Fig. 3e**).

Similarly, Pyr-sulf induces the upregulation of the same genes but with the upregulation of more heat shock response genes (*HSPA5*, *HSPA4*, *HSP90AA1*) and also *SQSTM1* (Sequestosome 1, a gene that encodes for a multifunctional protein that binds ubiquitin and regulates activation of NF-κB signalling pathway), genes predicted to be directly activated by ATF4 and indirectly activated by albumin (ALB), acyl-CoA oxidase 1 (ACOX1) and angiopoietin 2 (ANGPT2) (**Fig. 3f**). Overall, Pyr-sulf seems to be contributing to LUHMES neurospheroids cellular homeostasis (**Fig. 3f**).

Circulating (poly)phenol metabolites neuroprotection acts by transcriptional modulation of apoptosis and Nrf2 target genes

Due to the neuroprotective potential of both Cat-sulf and Pyr-sulf towards MPP^+ lesion (**Fig. 1d** and **1e**), and their differential role in modulating several genes involved in central canonical pathways in LUHMES cells (**Fig. 2** and **3**), changes in gene expression of specific genes involved in apoptosis regulation and in Nrf2-mediated response were evaluated in the PD 3D cell model.

The mRNA levels of major apoptosis players, the anti-apoptotic BCL2 and the pro-apoptotic BAX, were significantly modulated in neuroprotection conditions. After 6h of MPP^+ lesion, the pre-incubation with Ide, Cat-sulf and Pyr-sulf leads to an increase in BCL2 mRNA levels (**Fig. 4a**). However, after 24h of lesion, metabolites pre-incubation is no longer able to modulate BCL2 levels but leads to a significant decrease in BAX mRNA levels (**Fig. 4a**). BCL2/BAX ratio reflects the counteraction of Ide, Cat-sulf and

Pyr-sulf towards MPP^+ lesion, being significantly increased for all conditions at 6h of lesion, and lasting in time (24h of lesion) for Ide and Pyr-sulf (**Fig. 4a**).

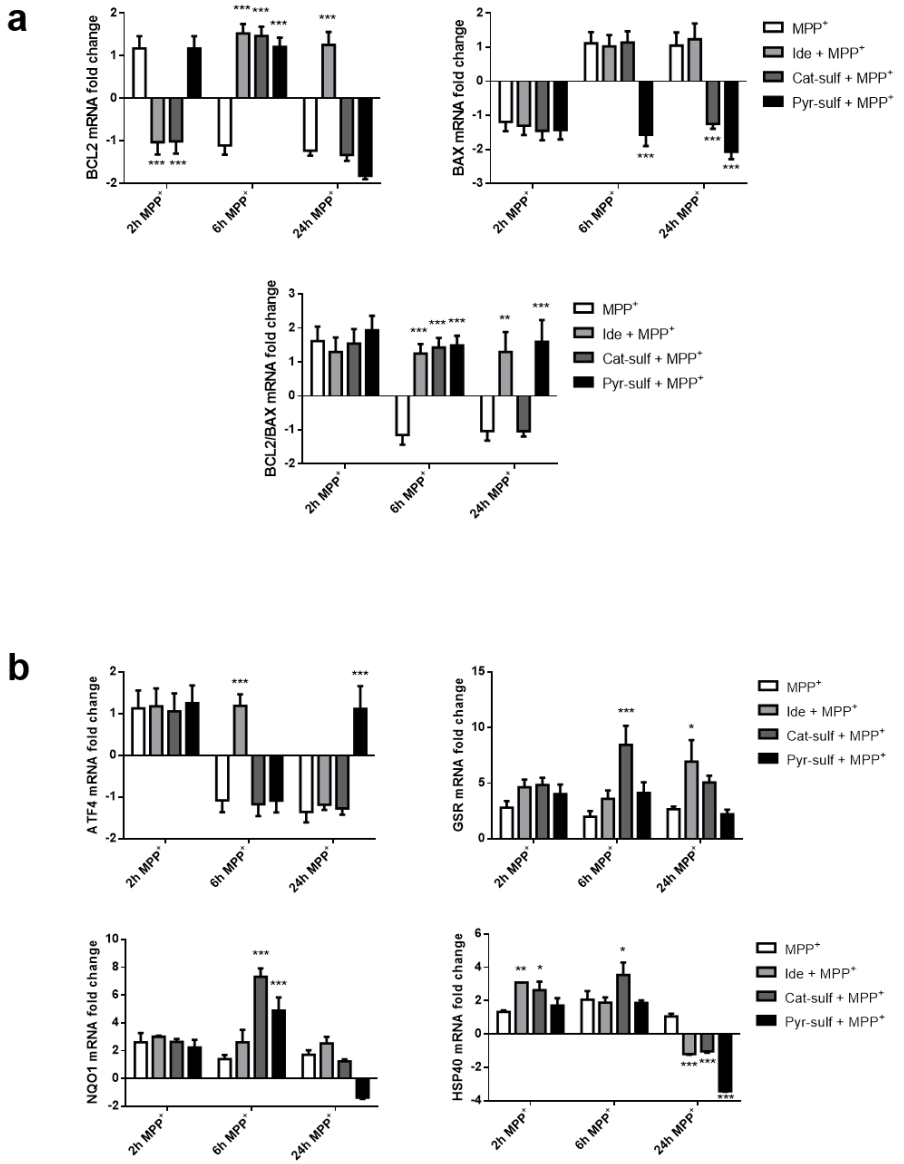

Fig. 4. Circulating (poly)phenol metabolites neuroprotective potential through genes modulation in LUHMES neurospheroids treated with MPP^+. (**a**) RT-qPCR analysis of apoptosis-related genes: *BCL2*, *BAX* and the ratio *BLC2/BAX* for neurospheroids pre-incubated with Ide, Cat-sulf or Pyr-sulf and then challenged for 2, 6 or 24h with MPP^+. (**b**) RT-qPCR analysis of Nrf2 target genes: *ATF4*, *GSR*, *NQO1* and *HSP40* for 2, 6 or 24h with MPP^+. Statistical differences are denoted as *p<0.05, **p<0.01, and ***p<0.001 relative to each time point of MPP^+ insult. Data are mean ± SEM of three independent cultures.

Nrf2 target genes *ATF4*, *GSR*, *NQO1* and *HSP40* were also significantly upregulated in the presence of the metabolites (**Fig. 4b**). After 2h of lesion with MPP^+, no significant alterations were denoted for mRNA levels of *ATF4*, *NQO1* and *GSR*. However, Ide and Cat-sulf lead to an increase in heat-shock protein 40 (*HSP40*) mRNA levels, which is sustained until 6h of lesion in Cat-sulf conditions (**Fig. 4b**). *ATF4* levels decreased with lesion time, being this trend only counteracted by Ide, at 6h, and by Pyr-sulf later in time (**Fig. 4b**). Moreover, at 6h of lesion, there is a significant increase in glutathione reductase (*GSR*) induced by Cat-sulf, and in NAD(P)H quinone oxidoreductase (*NQO1*) induced by Cat-sulf and Pyr-sulf (**Fig. 4b**). After 24h of MPP^+ lesion, Pyr-sulf seems to be coping with cells to deal with the insult, responding to the effects caused by the toxicant, helping to restore the mRNA levels of chaperones, phase I and II enzymes and antioxidant proteins (**Fig. 4b**).

Discussion

The complexity of age-related disorders, like PD, makes the identification of novel interventions particularly challenging. Current therapies only alleviate physical complications of advanced stages of the disease, without being able to treat it. The possibility to prevent or slow the progression of neurodegenerative diseases through diet would have huge social and economic repercussions.

(Poly)phenol-rich foods, like fruits and vegetables, have been described in last years to present brain health benefits with impacts in neurodegeneration[14, 15, 37], which has gathered researchers attention. In fact, different food and beverages seem to originate some common breakdown products which can be found in circulation and excreted in urine in human volunteers[25-29]. For instance, berries, which are rich in anthocyanins, hydroxybenzoic acids and hydroxycinnamic acids, when digested, originate some (poly)phenol metabolites in circulation that are common with the ones found in circulation after the consumption of other completely different food matrixes, like black tea, which is rich in flavonols like catechin and epicatechin[26, 29]. In fact, structurally diverse substrates yield essentially the same set of microbiota-derived (poly)phenol metabolites. These circulating (poly)phenol metabolites are mainly simple phenolic sulfates[26], where two of the most abundant detected after the consumption of a berry mixture puree were catechol-*O*-sulfate (Cat-sulf), and pyrogallol-1-*O*-sulfate and pyrogallol-2-*O*-sulfate (Pyr-sulf), in which some volunteers reach concentrations as high as 20μM[26]. As such, these bioavailable (poly)phenol metabolites can constitute true effectors in cellular and molecular mechanisms of the disease never explored before for a neurodegenerative disorder like PD.

Previous work from our lab have demonstrated that both Cat-sulf and Pyr-sulf can be transported across BBB endothelia at physiological concentrations[30], holding great promise as selective targets with protective potential for brain cells, with ultimate impact in the prevention of neurodegeneration. In fact, these metabolites proven to be protective in different cell models with increasing complexity, recapitulating common hallmarks of major neurodegenerative diseases, namely, oxidative stress at BBB level, neuronal glutamatergic excitotoxicity, oxidative stress in a three-dimensional cell model

of neurons and astrocytes, and neuroinflammation in microglial cells[30]. Their potential towards PD, however, was never reported before.

Differentiated LUHMES neurospheroids comprise a valuable tool for the assessment of (poly)phenol metabolites potential. In this work in particular, it was assessed (poly)phenol metabolites potential against one of the most important hallmarks of PD which is the death of dopaminergic neurons, in here recapitulated by a dopaminergic lesion caused by MPP^+[38]. The three-dimensional architecture of cells offer physiological advantages compared with two-dimensional cultures, mimicking better the brain environment complexity, presenting cell-cell interactions. Moreover, LUHMES differentiated neurospheroids were enriched in dopaminergic neurons, which expressed DAT, TH and βIII-tubulin. Some GFAP-positive cells were also observed at 7d of differentiation, which can suggest that, in a 3D microenvironment, LUHMES cells may be able to be differentiated into glial lineages. A consistent differentiated phenotype was obtained after 7d of differentiation of 3D culture, resulting in the irreversible conversion of LUHMES neuronal precursors into a dopaminergic-enriched neuronal population. Differentiated neurospheroids showed a higher number of mature neurons with functional synaptic terminals able to respond to depolarizing stimuli (data not shown), highlighting the fact that LUHMES neurospheroids synaptic terminals are able to fuse the synaptic vesicles with the plasma membrane and perform exocytosis and neurotransmitters release. A variety of spontaneous firing patterns was also identified in individual cells of LUHMES neurospheroids (data not shown), suggesting the presence of both neurons and astrocytes, since the latter can also present spontaneous Ca^{2+} transients, although with significant lower frequencies compared to neurons[39, 40]. This observation reinforces our results showing presence of GFAP-positive cells homogenously distributed across the neurospheroids, as well as the increased protein and mRNA levels of astrocytic markers along differentiation.

LUHMES differentiated neurospheroids responded to MPP^+ lesion, as already reported before[32, 38], comprising a disease-relevant cell model to study the potential of (poly)phenol metabolites towards a dopaminergic lesion with relevance to PD. Although, we observed that both Cat-sulf and Pyr-sulf alone presented a slight toxicity at the

circulating concentrations to LUHMES neurospheroids. Such observation, may comprise a hormetic phenomenon: exposures to low doses of certain phytochemicals (which can be slightly toxic) pre-condition the cells to greater and later insults[41]. Moreover, we confirmed the neuroprotective effect of these (poly)phenol metabolites in LUHMES neurospheroids challenged with MPP^+. In hormesis situations, the cells' machinery is triggered through the activation of different alert mechanisms such as the modulation of pathways involved in stress response, which will help the cells to cope with further damages[42]. To test our hypothesis, a PCR array targeting major signal transduction pathways was designed and Cat-sulf and Pyr-sulf effects assessed in terms of gene expression.

Cellular signaling pathways and molecular mechanisms that mediate hormetic responses typically involve enzymes such as kinases and deacetylases, and transcription factors such as Nrf2 and NF-κB. In such conditions, cells increase their production of several proteins including growth factors, phase 2 and antioxidant enzymes, and protein chaperones[42]. In fact, Nrf2 signaling pathway emerged as the top canonical pathway modulated by these circulating (poly)phenol metabolites, being more activated by Pyr-sulf. The Nrf2 transcription factor, by binding to antioxidant response elements (AREs), leads to the expression of antioxidant and detoxifying enzymes, which will protect the cells against oxidative damage and will provide protection against exogenous agents through phase II enzymes activity[43, 44]. Recent reports start evidencing the potential of dietary (poly)phenols, not its circulating metabolites, towards Nrf2[45-48], even though the detailed mechanism of action is not yet fully elucidated. There are some evidences of two mechanisms of Nrf2 activation by (poly)phenols: (i) direct, through the formation of a semiquinone radical which will activate Nrf2 by modifying Nrf2 inhibitor (Keap1) thiols; and (ii) indirect, through the activation of several kinase pathways resulting in the phosphorylation of Nrf2 and subsequent induction Nrf2-dependet genes. Quinones derived from catechol, resorcinol and hydroquinone have been reported to dissociate Nrf2 from Keap1 through a direct mechanism[45]. Moreover, some catechol-derived quinones like levodopa, carnosol[49], epigallocatechin 3-gallate[50] or quercetin[51] can provide protective potential through the selective alteration of Keap1

and subsequent activation of Nrf2 pathway, without presenting extensive quinoprotein formation, which can be deleterious. We may suggest that a stronger effect of Pyr-sulf in the Nrf2 canonical pathway, comparatively to Cat-sulf, could reflect a greater capacity of this catechol-derived metabolite in stabilization of Nrf2 and consequent disruption of Nrf2/Keap1 interaction, leading to a more pronounced transcriptional activity of AREs[45].

A stronger effect of Pyr-sulf, comparatively to Cat-sulf, towards NF-κB signaling pathway was observed in LUHMES neurospheroids, which is in accordance with our previous results[30]. In LPS-stimulated microglia, Pyr-sulf was able to dramatically reduce TNF-α levels caused by LPS and also modulated NF-κB nuclear translocation; by opposition, Cat-sulf was unable to exert such protective potential towards inflammation[30]. Although we are aware of differential NF-κB regulation in microglia and neuronal cells, these observations can give us clues about a putative common mechanism of action with beneficial effects in two different brain cell settings. The impact of catechol-type (poly)phenols in NF-κB signaling pathway is starting to be elucidated. For instance, piceatannol (3,3',4'5-tetrahydroxystilbene) derived from grapes, rhubarb and sugarcane, suppressed NF-κB pathway in MCF-10A cells [52]. The authors speculate that an electrophilic quinone is formed as a consequence of oxidation of catechol moiety of piceatannol through direct interaction with critical cysteine thiols of IKKβ [the activating kinase of NF-κB inhibitor (IκBα)] thereby inhibiting its catalytic activity[52]. Moreover, chlorogenic acid and caffeic acid, phenolic compounds found in coffee, also with a catechol moiety, significantly suppressed NF-κB transcriptional activity, nuclear translocation of the p65 subunit, and phosphorylation of IκB kinase (IKK) in Caco-2 cells[53]. The authors argue that the functional moiety responsible for the anti-inflammatory effects in both phenolic compounds was the catechol group, which allows scavenging of intracellular ROS, thereby inhibiting H_2O_2-induced IL-8 production via suppression of pyruvate dehydrogenase kinase (PKD)-NF-κB signaling in human intestinal epithelial cells[53]. However, such approaches did not took advantage of circulating metabolites derived from dietary (poly)phenols, like Cat-sulf and Pyr-sulf, in more advanced cell models with ultimate translation to disease.

The tumor suppressor protein p53 and the p53 signaling pathway were predicted to be activated in LUHMES neurospheroids in the presence of both (poly)phenol metabolites. p53 plays a major role in inhibition of angiogenesis and, upon damage, it can activate DNA repair proteins, induce growth arrest by holding the cell cycle, or initiate apoptosis through BAX, BCL2 and caspase 3 induction, for instance[54]. These results could comprise an adaptive reaction of the cells, as a reflex of a neurohormetic effect: by inducing an adaptive response at sub-toxic doses, such compounds may protect against stronger insults[41]. Our previous data already reported a neurohormetic potential of (poly)phenol components resulting from *in vitro* digestion of blackberries through the activation of p53 signaling[55]. Such (poly)phenol digested components, despite of being immersed in a complex mixture, represent a more physiological *in vitro* approach to study nutritionally-relevant effects than the usage of parent dietary (poly)phenols which are not bioavailable nor bioaccessible[55]. The work presented here represents a step forward once bioavailable (poly)phenol metabolites, at circulating concentrations, significantly modulated p53 signaling pathway in LUHMES neurospheroids, validating the neurohormetic potential that (poly)phenols resulting from berries consumption could bear.

The significant effects of (poly)phenol metabolites in ATF4, both an inducer and a target of Nrf2, were here evidenced, emerging as an upstream regulator with ultimate beneficial effects in terms of cell homeostasis and viability. Our previous work have already highlighted the pivotal role of this transcription factor in response to (poly)phenol components resulting from *in vitro* digestion of blackberries[55] and its potential towards neuroprotection. In fact, ATF4 upregulation has already been described to protect against neuronal death in cellular models of PD[56] and can comprise a mechanism by which Cat-sulf and Pyr-sulf can be neuroprotective.

Genes involved in apoptosis response, like *BCL2* and *BAX*, and targets genes of Nrf2 pathway, like *ATF4, NQO1*, *GSR* and *HSP40*, emerged as significantly upregulated by Cat-sulf and Pyr-sulf. Their key role in major canonical pathways and upstream regulators identified in our datasets made them suitable candidates to evaluate in

dopaminergic lesion conditions as putative intervenient with neuroprotective potential. Both Cat-sulf and Pyr-sulf incubation lead to a significant increase in mRNA levels of the anti-apoptotic BCL2 and a decrease in BAX, the pro-apoptotic agent, counteracting the trend caused by the toxicant MPP^+. Modulation of apoptosis could be certainly one mechanism of action by which these circulating (poly)phenol metabolites can be neuroprotective in a PD-like scenario. Evidences also suggest that oxidative stress is a major factor contributing to the vulnerability of dopaminergic cells and beneficial effects of the induction of AREs (Nrf2-target genes) towards PD is already described[57]. For instance, the induction of gene expression of *NQO1* was shown to confer protection to dopaminergic neurons in both cell culture and animal models of PD [58]. The upregulation of *NQO1* observed here, caused by the pre-incubation with Cat-sulf or with Pyr-sulf, and after 6h of MPP^+ lesion, corroborates such observations. Additionally, a decrease in glutathione (GSH) levels is one of the earliest biochemical alterations detected in association with PD and GSH itself regulates dopaminergic cell death through a wide variety of homeostatic processes [59]. In fact, in the *substantia nigra* of PD patients, there is a depletion of GSH with no change in oxidized glutathione (GSSG) levels[60]. By promoting the upregulation of the transcripts of glutathione reductase (GSR), enzyme which converts GSSG into GSH, (poly)phenol metabolites can be counteracting another important PD hallmark. Many neurodegenerative diseases, like PD, are 'proteinopathies' where toxic proteins aggregate and fibers that accumulate in an age-dependent process. The accumulation of insoluble protein aggregates with age implies a failure of the cellular protein quality control system, which consists of molecular chaperones, the ubiquitin-proteasome system and the autophagy-lysosome system [61]. In that sense, overexpression of Hsp70 chaperone is almost universally protective in models of PD[62-64]; however, the ATPase activity of Hsp70 (DnaK) is stimulated by its co-chaperone, Hsp40 (DnaJ), and these proteins often work in concert [65]. The upregulation of Hsp40 transcripts can be suggestive of an Hsp40-mediated activation of the regulatory mechanism of Hsp70 activity, with ultimate neuroprotective effects[55].

A better understanding of hormesis at the cellular and molecular levels is leading to novel approaches for the prevention and treatment of many different diseases [42]. We

observed that circulating (poly)phenol metabolites trigger several mechanisms which help the cells to cope with a later insult. Despite its multitude and complexity, it is believed that it may be possible to palliate some molecular symptoms of PD pathology with one single hit at the transcription factor Nrf2[66]. Circulating (poly)phenols metabolites, derived from human metabolism of different (poly)phenol-rich foods and which are BBB permeable, could fulfil this role. These circulating (poly)phenol metabolites modulate neuronal cells gene expression in order to tackle key points involved in PD never reported before for such metabolites. Moreover, as different sources of (poly)phenols give rise to the same circulating metabolites, it may be suggestive that such metabolites can be pivotal to keep our body health. In fact, such metabolites ability to tackle different cellular mechanisms, much likely to be involved in several chronic pathologies like neurodegenerative diseases, reinforce their potential as putative nutritional pleiotropic actors. Nevertheless, it may be only the first glimpse towards the understanding of the molecular effects on the benefits of the food we eat concerning age-related diseases like PD but, it can open doors for more mechanistic studies taking advantage of these circulating metabolites, physiological concentrations and disease-relevant cell models.

Material and Methods

Cell culture and 2D differentiation

Undifferentiated LUHMES cells (kindly provided by Dr. M. Leist) were routinely cultivated in proliferation medium, comprising Advanced DMEM/F12 (Gibco, Life Technologies) supplemented with 2mM L-Glutamine (Sigma-Aldrich), 1x N2 Supplement (Gibco) and 0.04 g/mL recombinant basic fibroblast growth factor (bFGF, R&D Systems). For propagation in monolayer, flasks (Nunc) were pre-coated with 50g/mL poly-L-ornithine (PLO, Sigma-Aldrich) and 1μg/mL fibronectin (Sigma-Aldrich).

3D stirred suspension culture

LUHMES cells were cultured in stirred suspension culture systems in a humidified atmosphere of 5% CO_2, at 37°C. Undifferentiated LUHMES cells were inoculated as single cell suspension in a silanized 125mL spinner vessel (Corning) equipped with a magnetic paddle impeller, at a density of 1.2 x 10^5 cell/mL in 125mL of proliferation medium. After 48h of aggregation, 70% of medium was changed to differentiation medium to induce neurospheroid cell differentiation in 3D. Differentiation medium comprised Advanced DMEM/F12 supplemented with 2mM L-Glutamine, 1x N2 Supplement, 1mM dibutyryl cAMP (Sigma-Aldrich), 2μg/mL tetracycline (Sigma-Aldrich), and 2ng/mL recombinant human glial cell line-derived neurotrophic factor (GDNF, R&D Systems). Replacement of 50% of culture medium by fresh differentiation medium was performed every 2-3 days. Cultures were maintained up to 14 days of differentiation. The agitation rate was increased along culture to avoid aggregate clumping and to control aggregate size (from initial 60rpm, to 70rpm at differentiation step initiation, and up to 80rpm by the end of the culture).

Neurospheroid monitoring along time

At different time points along culture progression, viability of differentiated neurospheroids was assessed using a fluorescence microscopy based method, the fluoresceine diacetate (FDA)/propidium iodide (PI) assay[67]. Moreover, cell proliferation was also assessed by fluorescence microscopy using Click-iT Plus EdU AlexaFluor™

488 Assay kit (Life Technologies). Neurospheroids sampling was performed along differentiation and neurospheroids disrupted in 0.1 M citric acid with 1% Triton X-100, at 37 °C, overnight, and nuclei stained with 0.1% crystal violet[68] and counted in a Fuchs–Rosenthal haemocytometer chamber. A sample size of at least 1 mL of culture was used to reliably determine cell concentration, neurospheroids counting and perform aggregate size measurements using ImageJ software.

Standard experimental setup

Differentiated neurospheroids (at 7 days of stirred differentiation) were collected and seeded in PLO-fibronectin coated 96, 24 or 12-well plates (Falcon) accordingly to following assays. After 24h, 200nM of Idebenone (positive control, provided by Grupo Tecnimede) or 5 µM of each (poly)phenol metabolite (catechol-sulfate, Cat-sulf or pyrogallol-sulfate, Py-sulf) was added and, after 24h of incubation, differentiation medium was carefully removed and new differentiation medium containing 5µM of MPP^+ (Sigma-Aldrich) was added to neurospheroids for different timepoints. Cell viability and ATP levels were assessed after 24h of injury, and mRNA levels of different targets at 0, 2h, 6h and 24h of injury with MPP^+.

Neurospheroids synaptic activity

Evoked synaptic activity of neurospheroids was assessed by the method of Gaffield 2006 [69] and already reported by us[70]. LUHMES neurospheroids were collected at 0 and 7 days of differentiation and seeded in PLO-fibronectin pre-coated glass coverslips, and incubated with 100mM KCl buffer (5mM HEPES-NaOH, pH 7.4; 10mM glucose; 2.5mM calcium chloride; 1mM magnesium chloride; 100mM potassium chloride; 37mM sodium chloride) for 5min. Afterwards, 100mM KCl buffer was removed and neurospheroids incubated with 10µM FM-1-43 dye (Invitrogen) in 5mM KCl buffer (5mM HEPES-NaOH, pH 7.4; 10mM glucose; 2.5mM calcium chloride; 1mM magnesium chloride; 5mM potassium chloride; 37mM sodium chloride) for 15min. Neurospheroids were washed for 1min with 5mM KCl buffer with ADAVASEP-7 (Sigma), followed by three washes with 5mM KCl buffer. Exocytosis was stimulated with 100mM KCl buffer and samples were visualized live using a fluorescence microscope (Leica DMI6000) in order to

monitor the decrease in fluorescence intensity over time. Fluorescence intensity was measured using ImageJ software.

Spontaneous synaptic activity of LUHMES neurospheroids was assessed by their response in calcium assay[71]. Neurospheroids with 7 days of differentiation were incubated with 1x Fluo-4 Direct calcium reagent (Invitrogen) for 30 min at 37˚C, 5% CO_2 and for 15 min at RT. Samples were then imaged live using spinning disk microscopy (Nikon Eclipse Ti-E, confocal scanner: Yokogawa CSU-x1). Fluorescence change over time is defined as ΔF/F0 = (F-F0)/F0, where F is the fluorescence at any time point, and F0 the baseline fluorescence determined by baseline fitting across the whole movie for each cell using SparkMaster plugin of ImageJ software.

Immunofluorescence microscopy

Neurospheroids were collected at 7 days of differentiation and processed for immunofluorescence staining[70]. Briefly, neurospheroids were fixed in 4% (w/v) paraformaldehyde (Sigma-Aldrich) solution in PBS with 4% (w/v) sucrose and processed directly for immunostaining. The primary antibodies used for cell characterization were anti-βIII-tubulin (βIII-tub, Millipore), anti-glial fibrillary acidic protein (GFAP, DAKO) and anti-tyrosine hydroxylase (TH, Santa Cruz Biotechnology). The secondary antibodies used were goat anti-mouse IgG-AlexaFluor 488 and goat anti-rabbit IgG AlexaFluor 594 (Life Technologies). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Life Technologies). Preparations were visualized using a point-scan confocal (Leica SP5) microscope. Merge between channels, maximum z-projections, and orthogonal projections, as well as linear brightness and contrast adjustment of the images were created using ImageJ software.

Western blot

Neurospheroids were lysed in TX-100 lysis buffer (50mM Tris, 5mM EDTA, 150mM NaCl, 1% Triton X-100 and 1x complete protease inhibitors cocktail (Roche)) and protein quantification was carried out using the Micro BCA™ Protein Assay kit (Thermo Fisher). Protein samples were subjected to a gel electrophoresis using a NuPAGE 4-12% Bis-Tris Gel with MES running buffer (Invitrogen) and electrophoretically transferred to

polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% non-fat dried milk powder in 0.1% Tween-20 in PBS (blocking solution) for 1h, followed by incubation with primary antibodies overnight at 4°C, diluted in blocking solution and then with secondary antibodies (horseradish peroxidase-conjugated, ECL anti-mouse IgG or anti-rabbit IgG; GE Healthcare) diluted 1:5000 in blocking solution, for 2h at RT. Anti-α-tubulin or anti-GAPDH antibodies were used as control to confirm equal loading of total protein. Membranes were developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and visualized using a ChemiDoc™ XRS+ System (BioRad). Primary antibodies used for protein detection were anti-βIII-tubulin (Millipore), anti-synaptophysisn (Syn, Millipore), anti-GFAP (DAKO), anti-TH (Santa Cruz), anti-Nestin (Millipore), anti-GLT1 (Millipore) and anti-Pitx3 (Abcam).

Gene expression

Neurospheroids total RNA was extracted with High Pure RNA Isolation kit (Roche) and quantified using NanoRop 2000c (ThermoScientific). Reverse transcription was performed with Transcriptor High Fidelity cDNA Synthesis kit (Roche), using Anchored-oligo(dT)18 Primer. Real-time quantitative PCR analysis (RT-qPCR) was performed as described[72]. RT-qPCR analysis was performed in a LightCycler 480 Multiwell Plate 96 (Roche), according to LightCycler 480 SYBR Green I Master kit (Roche). cDNA was diluted 1:2 and primers were used at 5□M, in 20□L reaction; each sample was performed in triplicate. The list of used primers and sequence is presented in **Supplementary Table S1**. RealTime ready assays from Universal Probe Library (Roche) were used with forward and reverse primers (400 nM) and fluorescently labeled hydrolysis probes (200 nM) lyophilized in a Custom Panel 384 (configuration no. 100127094, Roche, **Supplementary Table S2**) and were performed according to manufacturer's instructions. Real-time polymerase chain reaction analysis were performed in a final volume of 10 □l using the LightCycler® 480 Probes Master (Roche). Cycles threshold (Ct's) and melting curves were determined using LightCycler 480 software, version 1.5 (Roche) and results were processed using the $2^{-\Delta\Delta}$Ct method for relative gene expression analysis[72, 73]. Changes in gene expression were normalized using the

housekeeping genes *RPL22*, in the case of RT-qPCR, and *GAPDH*, *B2M* and *ACTB*, in the case of RealTime Ready assay.

Functional enrichment and pathway analysis

Ingenuity® Pathway Analysis (IPA, Ingenuity Systems, USA) was used for functional enrichment analysis. The Core Analysis function included in IPA was applied to analyze the lists of differentially expressed genes identified in of RealTime Ready PCR data. Based on the list of up and downregulated genes, IPA performs functional enrichment analysis in order to identify the biological processes and functions over-represented in a given list of genes. In addition to this, based on information stored in the Ingenuity® Knowledge Base, upstream regulator analysis was performed using IPA software. The upstream regulator analysis tool of IPA is based on the examination of the known targets of each transcription regulator in the list of differentially expressed genes, and it compares their direction of change to what is expected from the literature.

Cell viability assays

Neurospheroids were collected from spinner vessels at day 7 of differentiation and were distributed in pre-coated 96-well plates, with 15 neurospheres/well in differentiation medium. Six wells were used per test condition. As endpoints, metabolic activity was assessed by Presto Blue assay (Invitrogen), as a measure of resazurin conversion, and intracellular ATP levels by CellTiter Glo (Promega), by luminescence, according to manufacturer's instructions. Final cell viability (metabolic capacity) and intracellular ATP levels were calculated as a percentage relatively to control cells (untreated neurospheroids).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software. RT-qPCR, ATP fold change, neurotoxicity and neuroprotection data are mean ± SEM from at least three independent experiments (independent spinner vessels) performed with technical replicates. One-way ANOVA analysis with Tukey's post multiple comparison test was performed to assess statistical differences between conditions in all experiments.

Significance of the top enriched categories in IPA was tested by the Fisher Exact test *p*-value. The prediction algorithm of IPA calculates a z-score, and it is designed to reduce the chance that random data would generate significant predictions.

Acknowledgments

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Author's contributions

IF designed the research, performed the laboratory experiments, performed general data analysis and interpretation, and prepared the manuscript; APT contributed to the 3D LUHMES model implementation and design of drug assays and performed the laboratory experiments related with model characterization/validation; JP performed the laboratory experiments related with RT-qPCR and performed the statistical analysis; ML contributed to data interpretation and critically revised the manuscript; CB designed the research, contributed to data analysis and interpretation and critically revised the manuscript; CNS designed the research, contributed to data analysis and interpretation and critically revised the manuscript.

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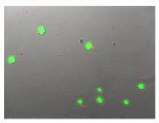
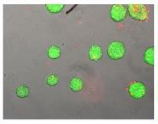
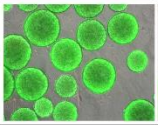
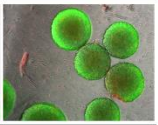
Supplementary material

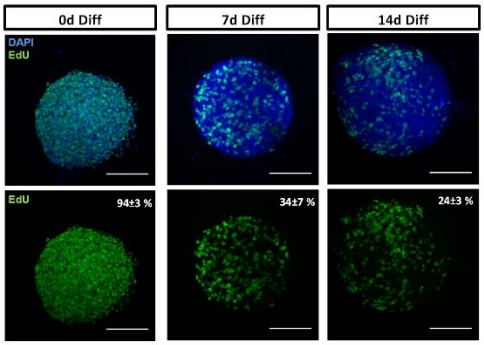
a

Inoculum

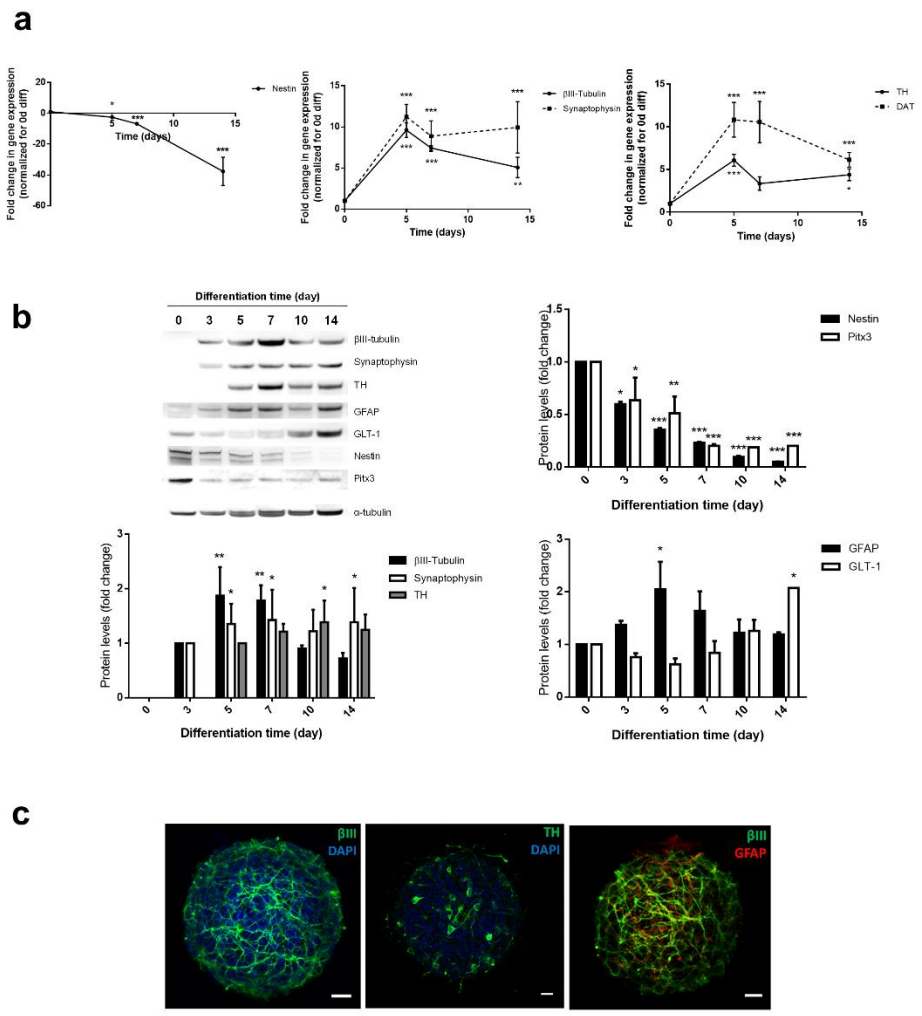
Differentiation Medium

Agg	0d	7d	14d Diff
			
Neurosphere diameter (µm)	125 ± 35	277 ± 81	326 ± 76
Neurosphere/ mL	608 ± 88	411 ± 143	264 ± 99
Cells/ neurosphere	1125 ± 765	6207 ± 1964	39980 ± 3950

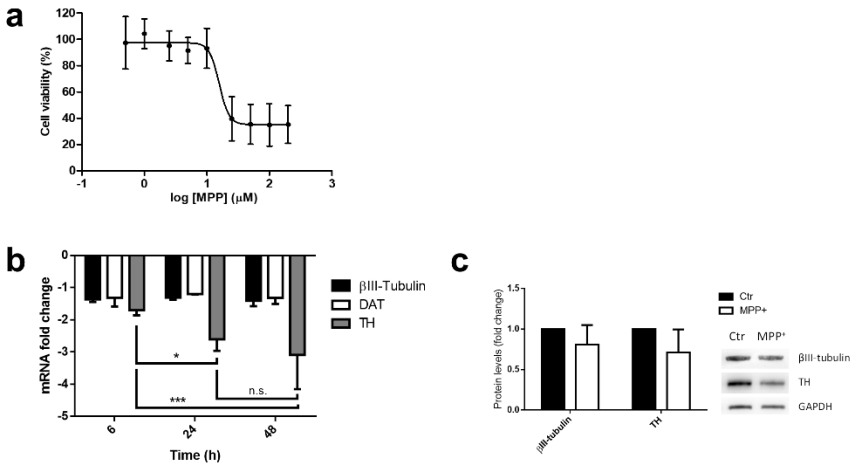
b



Supplementary Fig. S1 LUHMES neurospheroids cell culture characterization along time. Cells were inoculated in stirred suspension culture systems and aggregated (Agg) for 2 days. Dopaminergic differentiation was induced by removing FGF from medium and by introduction of GDNF, cAMP and tetracycline during 14 days (Differentiation Medium). (**a**) Representative images of the culture status at the indicated differentiation (Diff) days: fluorescent live/dead assay using fluorescein diacetate (FDA) for identification of live cells (green) and propidium iodide (PI) for identification of dead cells (red). (**b**) Representative images of neurosphere proliferation status at the indicated differentiation (Diff) days and percentage of proliferative cells (EdU positive cells): EdU labeling of proliferative cells (green) and nuclei counterstain with DAPI (blue). Data are mean ± SD of three independent cultures.



Supplementary Fig. S2 LUHMES neurospheroids culture neural population characterization. (**a**) RT-qPCR analysis of Nestin, βIII-tubulin, synaptophysin, TH and DAT gene expression; fold increase in gene expression of neurospheroids along culture time normalized for 0d of differentiation. Statistical differences are donated as ***p<0.001, **p<0.01 and *p<0.05 relative to 0d differentiation. (**b**) Detection of βIII-tubulin, synaptophysin, TH, Pitx3, GFAP, GLT-1 and Nestin by Western blot analysis and corresponding fold change in protein levels obtained by densitometry normalized for 0d of differentiation; α-tubulin was used as loading control. Statistical differences are donated as *p<0.05, **p<0.01, ***p<0.001 and relative to 0d differentiation. (**c**) Immunofluorescence microscopy of neurospheroids (7d Diff); scale bar: 20μm; βIII-tubulin (green), TH (green), GFAP (red), and DAPI (blue).



Supplementary Fig. S3 Parkinson's disease 3D cell model. Differentiated LUHMES neurospheroids (7d Diff) were submitted to a dopaminergic neurotoxicant, 1-methyl-4-phenylpyridinium (MPP^+). (**a**) Dose-response curve of 0.5-100μM MPP^+ induced lesion for 24 hours with respective IC_{50}, determined by Presto blue assay. (**b**) RT-qPCR analysis of βIII-tubulin, TH and DAT gene expression; fold change in gene expression of neurospheroids treated with 5μM MPP^+ after 6, 24 and 48h. Statistical differences are denoted as * $p<0.05$ or *** $p<0.0001$ as indicated. (**c**) Detection of βIII-tubulin and TH by western blot analysis and corresponding fold change in protein levels obtained by densitometry and normalized for control condition (no MPP^+). GAPDH was used as loading control. Data are mean ± SD of three independent cultures.

Supplementary Table S1 List of primers used for RT-qPCR analysis.

Gene	Cell type/Function	Primers forward (top) and reverse (bottom)
RPL22	Ribosomal protein L22 (housekeeping gene)	CACGAAGGAGGAGTGACTGG TGTGGCACACCACTGACATT
TUJ1	βIII-tubulin	GGGCCTTTGGACATCTCTTC CCTCCGTGTAGTGACCCTTG
TH	Tyrosine hydroxylase	AGCCCTACCAAGACCAGACG GCGTGTACGGGTCGAACTT
DAT	Dopamine transporter	ACCTTCCTCCTGTCCCTGTT CACCATAGAACCAGGCCACT
SYP	Synaptophysin	TTTGTGAAGGTGCTGCAATG GCTGAGGTCACTCTCGGTCT
NES	Nestin	TAAGGTGAAAAGGGGTGTGG GCAAGAGATTCCCTTTGCAG
GFAP	Glial fibrillary acid protein	AGAGAGGTCAAGCCCAGGAG GGTCACCCACAACCCCTACT
ATF4	Activating transcription factor 4	GGCTGGCTGTGGATGGGTTG CTCCTGGACTAGGGGGGCAA
GSR	Glutathione reductase	CGTGGAGGTGCTGAAGTTCTC TCATGGTCATGACTGGTAGCC
NQO1	NAD(P)H quinone dehydrogenase 1	TGACATATAGCATTGGGCACAC TTCTCCTCATCCTGTACCTCTT
HSP40	DnaJ (heat shock protein 40 kD)	AGGAAGGCCTAAAGGGGAGT AGGGATTTCTGCCACCGAAG
BCL-2	B-cell lymphoma 2	ATCGCCCTGTGGATGACTGAG CAGCCAGGAGAAATCAAACAGAGG
BAX	BCL-2 associated X protein	TGGAGCTGCAGAGGATGATTG GAAGTTGCCGTCAGAAAACATG

Supplementary Table S2 RealTime Ready Custom panel information. Assay ID, gene symbol (*H. sapiens*), alias and description according to https://configurator.realtimeready.roche.com/

Assay ID	Gene Symbol	Alias	Description
138446	ACSL3	ACS3; FACL3; PRO2194	acyl-CoA synthetase long-chain family member 3 [Source:HGNC Symbol;Acc:3570]
127578	ACSL4	ACS4; FACL4; LACS4; MRX63; MRX68	acyl-CoA synthetase long-chain family member 4 [Source:HGNC Symbol;Acc:3571]
119501	ACSL5	ACS2; ACS5; FACL5	acyl-CoA synthetase long-chain family member 5 [Source:HGNC Symbol;Acc:16526]
100816	AKT1	AKT; MGC99656; PKB; PKB-ALPHA; PRKBA; RAC; RAC-ALPHA	v-akt murine thymoma viral oncogene homolog 1 [Source:HGNC Symbol;Acc:391]
114661	ATF4	CREB-2; CREB2; TAXREB67; TXREB	activating transcription factor 4 (tax-responsive enhancer element B67) [Source:HGNC Symbol;Acc:786]
115129	ATF6	ATF6A	activating transcription factor 6 [Source:HGNC Symbol;Acc:791]
118103	ATG12	APG12; APG12L; FBR93; HAPG12	ATG12 autophagy related 12 homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:588]
125999	ATG5	APG5; APG5-LIKE; APG5L; ASP; hAPG5	ATG5 autophagy related 5 homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:589]
120541	ATG7	APG7-LIKE; APG7L; DKFZp434N0735; GSA7	ATG7 autophagy related 7 homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:16935]
102998	AXIN2	AXIL; DKFZp781B0869; MGC10366; MGC126582	axin 2 [Source:HGNC Symbol;Acc:904]
142318	BAX	BCL2L4	BCL2-associated X protein [Source:HGNC Symbol;Acc:959]
142759	BBC3	FLJ42994; JFY1; PUMA	BCL2 binding component 3 [Source:HGNC Symbol;Acc:17868]
100083	BCL2	Bcl-2	B-cell CLL/lymphoma 2 [Source:HGNC Symbol;Acc:990]
100085	BCL2A1	ACC-1; ACC-2; BCL2L5; BFL1; GRS; HBPA1	BCL2-related protein A1 [Source:HGNC Symbol;Acc:991]
100088	BCL2L1	Bcl-X; bcl-xL; BCL-XL/S; bcl-xS; BCL2L; BCLX; BCLXL; BCLXS; DKFZp781P2092	BCL2-like 1 [Source:HGNC Symbol;Acc:992]
100115	BECN1	ATG6; beclin1; VPS30	beclin 1, autophagy related [Source:HGNC Symbol;Acc:1034]
100122	BID	FP497; MGC15319; MGC42355	BH3 interacting domain death agonist [Source:HGNC Symbol;Acc:1050]
100135	BIRC3	AIP1; API2; c-IAP2; cIAP2; HAIP1; hiap-1; HIAP1; MALT2; MIHC; RNF49	baculoviral IAP repeat-containing 3 [Source:HGNC Symbol;Acc:591]
146274	BMP2	BMP2A	bone morphogenetic protein 2 [Source:HGNC Symbol;Acc:1069]
146294	BMP4	BMP2B; BMP2B1; MCOPS6; OFC11; ZYME	bone morphogenetic protein 4 [Source:HGNC Symbol;Acc:1071]
110980	BTG2	MGC126063; MGC126064; PC3; TIS21	BTG family, member 2 [Source:HGNC Symbol;Acc:1131]
-	CALR	cC1qR; CRT; FLJ26680; RO; SSA	calreticulin [Source:HGNC Symbol;Acc:1455]
113395	CCL5	D17S136E; MGC17164; RANTES; SCYA5; SISd; TCP228	chemokine (C-C motif) ligand 5 [Source:HGNC Symbol;Acc:10632]

142502	CCND1	BCL1; D11S287E; PRAD1; U21B31	cyclin D1 [Source:HGNC Symbol;Acc:1582]
101384	CCND2	KIAK0002; MGC102758	cyclin D2 [Source:HGNC Symbol;Acc:1583]
144097	CDKN1A	CAP20; CDKN1; CIP1; MDA-6; P21; p21CIP1; p21Cip1/Waf1; SDI1; WAF1	cyclin-dependent kinase inhibitor 1A (p21, Cip1) [Source:HGNC Symbol;Acc:1784]
100855	CDKN1B	CDKN4; KIP1; MEN1B; MEN4; P27KIP1	cyclin-dependent kinase inhibitor 1B (p27, Kip1) [Source:HGNC Symbol;Acc:1785]
-	CEBPD	C/EBP-delta; CELF; CRP3; NF-IL6-beta	CCAAT/enhancer binding protein (C/EBP), delta [Source:HGNC Symbol;Acc:1835]
113740	CPT2	CPT1; CPTASE	carnitine palmitoyltransferase 2 [Source:HGNC Symbol;Acc:2330]
141077	CSF1	M-CSF; MCSF; MGC31930	colony stimulating factor 1 (macrophage) [Source:HGNC Symbol;Acc:2432]
116795	DAB2	DOC-2; DOC2; FLJ26626	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila) [Source:HGNC Symbol;Acc:2662]
100355	DDIT3	CEBPZ; CHOP; CHOP-10; CHOP10; GADD153; MGC4154	DNA-damage-inducible transcript 3 [Source:HGNC Symbol;Acc:2726]
115809	DNAJC3	FLJ21288; HP58; P58; P58IPK; PRKRI	DnaJ (Hsp40) homolog, subfamily C, member 3 [Source:HGNC Symbol;Acc:9439]
143550	EGFR	ERBB; ERBB1; HER1; mENA; PIG61	epidermal growth factor receptor [Source:HGNC Symbol;Acc:3236]
148159	EMP1	CL-20; EMP-1; TMP	epithelial membrane protein 1 [Source:HGNC Symbol;Acc:3333]
103087	FABP1	FABPL; L-FABP	fatty acid binding protein 1, liver [Source:HGNC Symbol;Acc:3555]
145362	FAS	ALPS1A; APO-1; APT1; CD95; FAS1; FASTM; TNFRSF6	Fas (TNF receptor superfamily, member 6) [Source:HGNC Symbol;Acc:11920]
-	FCER2	CD23; CD23A; CLEC4J; FCE2; IGEBF	Fc fragment of IgE, low affinity II, receptor for (CD23) [Source:HGNC Symbol;Acc:3612]
104340	FOSL1	FRA; fra-1; FRA1	FOS-like antigen 1 [Source:HGNC Symbol;Acc:13718]
117090	FTH1	FHC; FTH; FTHL6; MGC104426; PIG15; PLIF	ferritin, heavy polypeptide 1 [Source:HGNC Symbol;Acc:3976]
101471	GADD45A	DDIT1; GADD45	growth arrest and DNA-damage-inducible, alpha [Source:HGNC Symbol;Acc:4095]
115714	GADD45B	DKFZP566B133; GADD45BETA; MYD118	growth arrest and DNA-damage-inducible, beta [Source:HGNC Symbol;Acc:4096]
110864	GATA3	HDR; MGC2346; MGC5199; MGC5445	GATA binding protein 3 [Source:HGNC Symbol;Acc:4172]
147654	GCLC	GCL; GCS; GLCL; GLCLC	glutamate-cysteine ligase, catalytic subunit [Source:HGNC Symbol;Acc:4311]
114136	GCLM	GLCLR	glutamate-cysteine ligase, modifier subunit [Source:HGNC Symbol;Acc:4312]
111427	GSR	MGC78522	glutathione reductase [Source:HGNC Symbol;Acc:4623]
102005	HERPUD1	HERP; KIAA0025; Mif1; SUP	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 [Source:HGNC Symbol;Acc:13744]
142326	HES1	bHLHb39; FLJ20408; HES-1; HHL; HRY	hairy and enhancer of split 1, (Drosophila) [Source:HGNC Symbol;Acc:5192]
-	HES5	bHLHb38	hairy and enhancer of split 5 (Drosophila) [Source:HGNC Symbol;Acc:19764]
142325	HEY1	BHLHb31; CHF-2; CHF2; HERP2; HESR-1; HESR1; HRT-1; MGC1274; OAF1	hairy/enhancer-of-split related with YRPW motif 1 [Source:HGNC Symbol;Acc:4880]

112778	HEY2	bHLHb32; CHF1; GRIDLOCK; GRL; HERP1; HESR2; HRT2; MGC10720	hairy/enhancer-of-split related with YRPW motif 2 [Source:HGNC Symbol;Acc:4881]
137073	HEYL	bHLHb33; HEY3; HRT3; MGC12623	hairy/enhancer-of-split related with YRPW motif-like [Source:HGNC Symbol;Acc:4882]
110977	HMOX1	bK286B10; HO-1; HSP32	heme oxygenase (decycling) 1 [Source:HGNC Symbol;Acc:5013]
110865	HSP90AA1	FLJ31884; HSP86; Hsp89; HSP89A; Hsp90; HSP90A; HSP90AA2; HSP90N; HSPC1; HSPCA; HSPCAL1; HSPCAL4; HSPN; LAP2	heat shock protein 90kDa alpha (cytosolic), class A member 2 [Source:HGNC Symbol;Acc:5256]
100489	HSP90B1	ECGP; GP96; GRP94; TRA1	heat shock protein 90kDa beta (Grp94), member 1 [Source:HGNC Symbol;Acc:12028]
110730	HSPA4	APG-2; HS24/P52; hsp70; hsp70RY; MGC131852; RY	heat shock 70kDa protein 4 [Source:HGNC Symbol;Acc:5237]
110805	HSPA5	BiP; FLJ26106; GRP78; MIF2	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa) [Source:HGNC Symbol;Acc:5238]
100945	ICAM1	BB2; CD54; P3.58	intercellular adhesion molecule 1 [Source:HGNC Symbol;Acc:5344]
104631	ID1	bHLHb24; dJ857M17.1; ID	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein [Source:HGNC Symbol;Acc:5360]
110609	IFNG	IFG; IFI	interferon, gamma [Source:HGNC Symbol;Acc:5438]
117683	IFRD1	PC4; TIS7	interferon-related developmental regulator 1 [Source:HGNC Symbol;Acc:5456]
144798	IRF1	IRF-1; MAR	interferon regulatory factor 1 [Source:HGNC Symbol;Acc:6116]
108043	JAG1	AGS; AHD; AWS; CD339; HJ1; JAGL1; MGC104644	jagged 1 [Source:HGNC Symbol;Acc:6188]
112383	LFNG	SCDO3	LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase [Source:HGNC Symbol;Acc:6560]
146268	LRG1	HMFT1766; LRG	leucine-rich alpha-2-glycoprotein 1 [Source:HGNC Symbol;Acc:29480]
106176	MAP3K1	MAPKKK1; MEKK; MEKK1	mitogen-activated protein kinase kinase kinase 1 [Source:HGNC Symbol;Acc:6848]
102930	MCL1	bcl2-L-3; BCL2L3; EAT; Mcl-1; MCL1-ES; mcl1/EAT; MCL1L; MCL1S; MGC104264; MGC1839; TM	myeloid cell leukemia sequence 1 (BCL2-related) [Source:HGNC Symbol;Acc:6943]
104396	MMP7	MMP-7; MPSL1; PUMP-1	matrix metallopeptidase 7 (matrilysin, uterine) [Source:HGNC Symbol;Acc:7174]
100977	MYC	bHLHe39; c-Myc; MRTL	v-myc myelocytomatosis viral oncogene homolog (avian) [Source:HGNC Symbol;Acc:7553]
142322	NOTCH1	hN1; TAN1	notch 1 [Source:HGNC Symbol;Acc:7881]
147227	NQO1	DHQU; DIA4; DTD; NMOR1; NMORI; QR1	NAD(P)H dehydrogenase, quinone 1 [Source:HGNC Symbol;Acc:2874]
113212	OLR1	CLEC8A; LOX-1; LOX1; LOXIN; SCARE1; SLOX1	oxidized low density lipoprotein (lectin-like) receptor 1 [Source:HGNC Symbol;Acc:8133]
101524	PCNA	MGC8367; PCNAAS	PCNA antisense RNA (non-protein coding) [Source:HGNC Symbol;Acc:37184]
104411	PPARD	FAAR; MGC3931; NR1C2; NUC1; NUCI; NUCII; PPARB	peroxisome proliferator-activated receptor delta [Source:HGNC Symbol;Acc:9235]
111252	PTCH1	BCNS; FLJ26746; FLJ42602; HPE7; NBCCS; PTC; PTC1; PTCH; PTCH11	patched 1 [Source:HGNC Symbol;Acc:9585]

105606	RAF1	c-Raf; CRAF; NS5; Raf-1	v-raf-1 murine leukemia viral oncogene homolog 1 [Source:HGNC Symbol;Acc:9829]
101596	RB1	OSRC; p105-Rb; pp110; pRb; RB	retinoblastoma 1 [Source:HGNC Symbol;Acc:9884]
117069	SLC27A4	ACSVL4; FATP4; IPS	solute carrier family 27 (fatty acid transporter), member 4 [Source:HGNC Symbol;Acc:10998]
101122	SOCS3	ATOD4; CIS3; Cish3; MGC71791; SOCS-3; SSI-3; SSI3	suppressor of cytokine signaling 3 [Source:HGNC Symbol;Acc:19391]
119555	SORBS1	CAP; DKFZp451C066; DKFZp586P1422; FLAF2; FLJ12406; KIAA1296; ponsin; R85FL; SH3D5; sh3p12; SORB1	sorbin and SH3 domain containing 1 [Source:HGNC Symbol;Acc:14565]
113560	SQSTM1	A170; OSIL; p60; p62; p62B; PDB3; ZIP3	sequestosome 1 [Source:HGNC Symbol;Acc:11280]
101180	STAT1	DKFZp686B04100; ISGF-3; STAT91	signal transducer and activator of transcription 1, 91kDa [Source:HGNC Symbol;Acc:11362]
147880	TNF	DIF; TNF-alpha; TNFA; TNFSF2	Tumor necrosis factor Precursor (TNF-alpha)(Tumor necrosis factor ligand superfamily member 2)(TNF-a)(Cachectin) [Contains Tumor necrosis factor, membrane form;Tumor necrosis factor, soluble form] [Source:UniProtKB/Swiss-Prot;Acc:P01375]
101266	TNFSF10	Apo-2L; APO2L; CD253; TL2; TRAIL	tumor necrosis factor (ligand) superfamily, member 10 [Source:HGNC Symbol;Acc:11925]
116591	TXNL4B	Dim2; DLP; FLJ20511	thioredoxin-like 4B [Source:HGNC Symbol;Acc:26041]
114835	TXNRD1	GRIM-12; MGC9145; TR; TR1; Trxr1; TXNR	thioredoxin reductase 1 [Source:HGNC Symbol;Acc:12437]
109914	ULK1	ATG1; ATG1A; FLJ38455; FLJ46475; Unc51	unc-51-like kinase 1 (C. elegans) [Source:HGNC Symbol;Acc:12558]
146965	WISP1	CCN4; WISP1c; WISP1i; WISP1tc	WNT1 inducible signaling pathway protein 1 [Source:HGNC Symbol;Acc:12769]
104468	WNT1	INT1	wingless-type MMTV integration site family, member 1 [Source:HGNC Symbol;Acc:12774]
104488	WNT2B	WNT13; XWNT2	wingless-type MMTV integration site family, member 2B [Source:HGNC Symbol;Acc:12781]
104494	WNT3A	MGC119418; MGC119419; MGC119420	wingless-type MMTV integration site family, member 3A [Source:HGNC Symbol;Acc:15983]
146266	WNT5A	hWNT5A	wingless-type MMTV integration site family, member 5A [Source:HGNC Symbol;Acc:12784]
47821	WNT6		wingless-type MMTV integration site family, member 6 [Source:HGNC Symbol;Acc:12785]

102065	B2M		beta-2-microglobulin [Source:HGNC Symbol;Acc:914]
143636	ACTB	PS1TP5BP1	actin, beta [Source:HGNC Symbol;Acc:132]
141139	GAPDH	G3PD; GAPD; MGC88685	glyceraldehyde-3-phosphate dehydrogenase [Source:HGNC Symbol;Acc:4141]

Chapter 5

Discussion

Neurodegenerative diseases such as Alzheimer's and Parkinson's are a global challenge and most of these diseases remain incurable and are strongly linked with aging populations. Dementias alone affect more than 7 million people in Europe and their care is estimated to cost EUR 130 billion a year (neurodegenerationresearch.eu/). The increasing burden of neurodegenerative diseases in the developed countries has fostered research in the fields of brain disease understanding and drugs development. However, the challenge facing the world of diagnosing, treating and caring for people affected by neurodegenerative diseases is extremely intimidating and no single country alone has the expertise or resources necessary to tackle all of the big questions in this area.

Potential alternatives to the current available drugs, that only alleviate major physical debilitating complications of disease progression, have emerged in the latest years. Nutrition can be one modifiable factor that could help to prevent, or at least retard, neurodegenerative diseases progression; it can be related with the adherence to more healthy dietary habits, valuing the intake of foods rich in (poly)phenols, like fruit and vegetables[1]. In fact, diets rich in fruits and vegetables have been recommended to reduce the risk of development of several chronic diseases[2]. Epidemiological and clinical studies have showed that dietary (poly)phenols are able to reduce the incidence and prevalence of such disorders, exhibiting remarkable multipotent ability to modulate several pathways[3-6]. The prevention and treatment of neurodegenerative disorders, characterized by mechanistic complexity, will require novel therapeutic strategies directed to multiple targets, such as (poly)phenols can do.

Berries are amongst the most promising sources of (poly)phenols, consistently associated with health benefits, containing a great diversity of compounds[7]. However, the precise contribution of dietary (poly)phenols and circulating (poly)phenol metabolites to human health is still not fully elucidated. Absorption and blood concentrations of some (poly)phenols is quite low, which can hamper the research in terms of understanding their effects in specific biomarkers of disease. The difficulty in demonstrating (poly)phenol true effects can also be justified by the uncertain metabolic fate that dietary (poly)phenols can have. In fact, it is necessary to identify the bioavailable metabolites

resulting from (poly)phenol ingestion through the diet, as well as their ability to overcome and/or interact with cellular barriers and reach target tissues. The work presented in this thesis is just the tip of the iceberg, concerning the point of view in how a nutritional approach, namely in exploring berries' potential towards brain health in neurodegeneration contexts, and how it can promote health benefits. And, of course, as any work in research, it raises more questions than answers that should be pursued.

Previous work from our lab has highlighted the importance of biochemical modifications that occur throughout berries' digestion and the ultimate impact that those comprise in terms of cellular bioactivities[8, 9]. However, the molecular mechanism behind berries neuroprotection effects remained to be elucidated. In my thesis work, we went deeper in understanding regarding the effects of bioaccessible components from berries resulting from *in vitro* digestion in terms of blood-brain barrier (BBB) endothelial permeability and the major cellular pathways modulated by them with potential neuroprotective effects (**Chapter 2**). Bioaccessible components resulting from *in vitro* digestion of berries have proven to be transported across the BBB, presenting cytoprotective effects at endothelial level. Moreover, they also showed to be neuroprotective in primary cultures of mouse cerebellar granule cells and in NT2-derived neurospheres, comprising a mixed population of neurons and astrocytes (**Chapter 2**). These bioaccessible components have, proven to be able to modulate central pathways involved in cell response, like mTOR, unfolded protein response and p53 signaling, with the rise of novel gene targets never reported before to be modulated by these components, like *ATF5*. Our work, together with others[10-13], represents a shift in mentality in the study of (poly)phenols' health benefits that has emerged in the last decade: by contemplating the digestion process, though in a simplistic way, we were able to overcome limitations of studying the compounds present in the raw food that we eat (dietary (poly)phenols), being in a better position to disclose the nutrition impact. Accumulating research over the years has thoroughly explored pure dietary (poly)phenols mode of action in age-related pathologies[14-16]. Such approach is of extreme importance but, unfortunately, is not always directly translatable to human's benefits in a nutritional perspective.

Within the active field of *in vitro* digestion in food research, the COST Action INFOGEST aimed to harmonize *in vitro* protocols simulating human digestion on the basis of physiologically inferred conditions. A harmonized static *in vitro* digestion method was implemented and validated across laboratories of the network[17, 18]. This harmonized model, despite of being more complex and more physiologically-relevant than the one used in my thesis, still presents some limitations, such as the lack of intestinal cells and microbiota contribution to the digestive process. Recent studies have already evidenced the impact of both to overall bioaccessibility and bioavailability of the compounds resulting from digestion[19-22]. Nevertheless, this COST Action effort represents a step forward with the aim to seek for a research in the field of nutrition every time more close to human physiology and metabolism. In my opinion, the paramount contribution of microbial metabolism should be comprehensively addressed in further studies regarding the attainment of bioaccessible components from food matrixes and their ultimate impact for human health.

In our lab we also have demonstrated that major bioavailable (poly)phenol metabolites resultant from berries consumption in humans are, in fact, derived from colonic microflora metabolism[23, 24]. From the human intervention study conducted where the volunteers took a berries-mix puree, biological samples (urine and blood) were analyzed. The analysis took into account a library of theoretically predicted metabolites which was rationally designed and the hits validated with chemically synthesized (poly)phenol metabolites[23, 24]. Some of the most abundant (poly)phenol metabolites detected in biological samples were simple phenolic sulfates, which in some volunteers reached concentrations as high as 20μM[24]. This human intervention study previously conducted in our lab was the first to identify and quantify these particular phenolic sulfates in human samples[24].Other researchers have identified the same phenolic sulfates in biological samples of volunteers that ingested other (poly)phenol-rich sources, such as cranberries, mango and tea[25-27]. It seems that different food matrixes, rich in different dietary (poly)phenols, once metabolized, give rise to the same breakdown products. Such bioavailable (poly)phenol metabolites offer than an

unexplored potential as true circulating molecules with putative effector potential in target tissues.

As such, in my thesis, we unveiled the power of circulating (poly)phenol metabolites in terms of BBB permeability and protective effects towards neurodegeneration and neuroinflammation (**Chapter 3**). We observed that all the phenolic sulfates studied were able to be transported across a simplified model of the BBB at circulating concentrations, although in different extent. Such differences in endothelial transport were speculated to be related with differences in chemical structure of these metabolites and/or putative interactions with efflux transporters. Moreover, these phenolic sulfates were not only able to be uptaken by endothelial cells, but also to be biotransformed into new (poly)phenol metabolites with unexplored potential. What are the bioactivities of these new cellular metabolites we do not know but certainly it would be of extreme importance to study them thoroughly once those are the ones that ultimately could reach brain cells. Chemical synthesis of these cellular (poly)phenol metabolites, mainly glutathione and acetylcysteine derivatives, should be pursued and neuroprotective potential assessed in relevant cell models of neurodegenerative diseases.

As a key tool used along this thesis, we took advantage of a validated, though simplified, *in vitro* model of the BBB (**Chapters 2 and 3**). *In vivo*, the BBB is much more than an endothelial monolayer, comprising a complex interface composed by several cell types, working in concert. As such, it would be very interesting to compare our results with the ones using more relevant BBB model systems, such as recently established quadrupole co-cultures with iPSC (BBB endothelial cells, perycites, astrocytes and neurons), more close to *in vivo* characteristics of a fully functional BBB, mimicking the neurovascular unit[28]. Increasing the complexity of the cell model would support our observations: if the same new cellular metabolites arose in more complex systems, that would justify that we would be one step closer to the real agents acting in the prevention of chronic diseases. As a final validation, of course, the logic step would be to evaluate *in vivo* BBB transport and metabolism with labelled compounds in animal models[29, 30]. Such knowledge would impact our understanding about (poly)phenols mode of action, with huge repercussions in terms of aging and age-associated diseases.

The two most abundant (poly)phenol metabolites and BBB endothelium permeable, catechol-sulfate (Cat-sulf) and pyrogallol-sulfate (Pyr-sulf), proven to be strong candidates to be further explored in a neuronal context. We observed that both were cytoprotective, at endothelial level, and neuroprotective towards oxidative insults in different cell models with increasing complexity (**Chapter 3**). Pyr-sulf also proven to be a strong attenuator of LPS-induced neuroinflammation in microglial cells, with the involvement of NF-κB pathway modulation. (Poly)phenols potential towards neuroinflammation and NF-κB pathway is already described for several dietary (poly)phenols, like resveratrol[31] and curcumin[32]. Our work, by taking advantage of (poly)phenol metabolites at circulating concentrations, highlighted a putative mechanism of action of bioavailable metabolites in neuroinflammation: Pyr-sulf, despite of not being able to prevent IκBα degradation and NF-κB phosphorylation, with consequent NF-κB nuclear translocation after 15 min of LPS exposure, helps to maintain NF-κB back to the cytoplasm by increasing the IkBα protein levels at 60 min (**Chapter 3**). The orchestrated and precise mechanism by which Pyr-sulf promotes such alterations in microglia along inflammation should be explored in deeper extent with, for instance, the evaluation of NF-kB target genes levels and also with the assessment of NF-kB nuclear translocation along time. By taking advantage of specific inhibitors of the NF-kB pathway or specific antisense oligonucleotides it would also allow a profounder understanding of the molecular mechanisms of these phenolic sulfates, to our knowledge never reported before. Due to the opposite effects of Cat-sulf and Pyr-sulf in neuroinflammation, metabolites which only differ by one hydroxyl group, it would be very interesting to study their role in other pathways involved in inflammatory responses like PKC (NFAT), MAPK/ERK, MAPK/JNK or JAK/STAT; in fact, we may guess that the most potent metabolite in NF-κB pathway may not be the most potent towards other pathways and such knowledge would help to better direct a future nutraceutical and/or pharmacological approach of these metabolites.

To close a circle of understanding of dietary (poly)phenols metabolites' neuronal benefits, the usage of a human cell model of a specific neurodegenerative disorder was attained. A three-dimensional cellular model enriched in dopaminergic neurons was

used, taking advantage of differentiable human neuronal precursor cells (**Chapter 4**). This model allowed us to propose potential pathways modulated by bioavailable (poly)phenol metabolites in a dopaminergic neuronal population, with potential impact to Parkinson's disease (PD). In fact, Pyr-sulf and Cat-sulf seem to act by a pre-conditioning effect: though being slightly toxic to cells, they are able to modulate central canonical pathways like Nrf2, p53 and NF-κB signaling in a three-dimensional environment, with relevance to neuroprotection). The up-regulation of Nrf2-target genes like *NQO1*, *GSR*, *ATF4* and *HSP40* and consequent modulation of such genes in the presence of a dopaminergic neurotoxicant, MPP^+, by Cat-sulf and Pyr-sulf, with ultimate protective effects to cells, evidenced one of the possible mechanisms by which the food that we eat can prevent and/or retard age-related neurodegenerative diseases – hormesis. The biochemical mechanisms of hormesis is not yet well understood but it is accepted that it can be triggered by low doses of toxins or other stressors which might activate the repair mechanisms of the cells or organism as a pre-conditioning effect. This repair process primes the cells to cope better with the insult and other damages that can arise[33].

The usage of MPP^+ in the 3D cell model highlights the dopaminergic-protective potential of bioavailable (poly)phenol metabolites with impact to PD pathology, in which the most affected brain cells are the dopaminergic neurons from *substantia nigra*[34]. Both Cat-sulf and Pyr-sulf proven to be neuroprotective against MPP^+ in different extent (**Chapter 4**). Of course, several other factors lead to PD complexity and are possibly more important than mitochondrial dysfunction and ROS production caused by MPP^+ to disease progression, such as α-synuclein aggregation and Lewy body formation[34]. As such, we should build up our data by the usage of other cellular models that can recapitulate better the disease phenotype, such as PD-patient derived iPSC dopaminergic cells or animal models with α-synuclein aggregates or even presenting α-synuclein familiar mutations. It would be very interesting to study if the pathways modulated by these metabolites in such scenarios and if they would be different and worth to explore. Nevertheless, our work reinforces the action of circulating (poly)phenols metabolites

resulting from (poly)phenol-rich sources' digestive process and microbiota metabolism to our brain health.

Most of our current knowledge on (poly)phenol research was obtained taking advantage of particular characteristics of cellular model systems with different degrees of complexity and their contribution in unveiling novel potential lead molecules is unquestionable. But, all models present limitations that should be considered. Pharmaceutical research drove the development of superior models from which the research in the field of human bioavailable (poly)phenol metabolites could benefit. The possibility to test their bioactivities in "mini-brains" settings, such as complex three-dimensional cultures of neurons, astrocytes, oligodendrocytes, pericytes, microglia and brain microvascular endothelial cells, would be of upmost importance. Systems like those would be much superior than, for instance, the most used cancer cell lines in 2D for (poly)phenol research, being closer to the modelling of the human brain as we may get. Several authors have attempted to obtain valuable 3D screening tools for drug testing to better understand how the function of a simple network of individual neurons adds up to the complexity of brain function, and how this knowledge can be applied to medicine[35-37]. Current state of art neural systems still present some challenges to overcome in order to give the right answers to the most complex diseases and (poly)phenols field of research can certainly benefit from that[38].

The work presented in this thesis focus on the nutritional impact of the food we eat to our brain health and the experimental approaches taken always considered conditions as close to physiological as possible. In all cell models used, circulating concentrations were applied, with all the benefits and limitations that such choice may encompass: by using physiological concentrations, the effects seen are more translatable to nutritional impact to our health, but it is always a more challenging approach once the low concentrations used can be masked by the overall setting. On the other hand, the (poly)phenol metabolites studied can also be regarded as putative novel therapeutics which can be encapsulated and provided as a drug. The bioavailability is an important issue and therefore the discussion about putative formulation of (poly)phenols and (poly)phenol metabolites is needed to overcome this problem,. As for any other drug, it

is important to address several questions about (poly)phenol metabolites behavior in human body like absorption, distribution, metabolism and excretion (ADME),; to overcome some bioavailability problems, it is necessary to investigate limitations in the absorption and metabolism of these compounds and their presence in the target tissues. In the case of neurodegenerative diseases, as already mentioned, it is of extreme importance the understanding of the ability of (poly)phenols to interact and/or cross the BBB and if they keep their bioactivity[39]. Several animal studies have attempted to disclose some of these questions but the answers gathered so far can also present problems due to huge differences in biology and metabolism of (poly)phenols comparing to humans.

Finally, the most reliable information on (poly)phenol bioactivity is indeed provided by clinical trials; however, their design should be further improved specially regarding the application of strict guidelines and high number of individuals. Thus, the standardization of clinical trials using natural compounds is of extreme importance for the rationale application of (poly)phenols in a nutraceutical and pharmacological context. Moreover, the absorption and metabolism of (poly)phenols involves multiple pathways which are subject to substantial inter-individual variation[40]. Some dietary (poly)phenols can be absorbed at intestinal level but most are hydrolyzed and metabolized by the colonic microbiota[41]. Phenolic sulfates studied in this thesis, as already mentioned, are colonic-derived metabolites, already reported by other authors to be present in human fluids[25, 26]. But in humans, different metabolizing phenotypes, or "metabotypes", have been reported, as for instance for ellagitannins[42], and such "metabotypes", which can be determined by the concentration and activity of intestinal carriers and post-absorptive metabolizing enzymes, and by the composition and activity of the colonic microbiota, will be strongly influenced by the genotype of the subject[43]. Thus, for the same dietary intake, exposure to bioactive metabolites can be markedly different between individuals. A personalized optimal nutrition can be based in the knowledge of human "metabotypes" and targeted recommendations may be given at each group levels[44].

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