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B.1 Basic Details

B1.1 Title:

From the gut to the AD brain: Identifying bioavailable therapeutic (poly)phenols and their metabolites for the treatment of Alzheimer's Disease

B.1.2 Abstract

AD accounts for 60-80% of all cases of dementia and leads to debilitating syndromes manifesting in memory loss and severe cognitive dysfunction. Failure of high-cost medicinal products in clinical trials has led scientists to turn their focus towards potential therapeutics provided by nature. Polyphenols are molecules derived from plants that can interfere with multiple disease mechanisms in AD. However, the remaining challenges for polyphenols as therapeutic candidates mainly relate to their bioavailability, as they are subject to metabolizations by gut microflora in order to be absorbed and exert their neuroprotective effect. Gut dysbiosis is a common feature of neurological disease, and therefore bioavailability of dietary (poly)phenols might be altered in patients with AD. Therefore, we propose to investigate bioavailable polyphenols metabolites under the conditions of AD-related dysbiosis using solely human-based *in vitro* technologies and assess their biological activity in state-of-the-art disease-specific AD models. For this aim, we will firstly characterize the differences in gut microbiome composition between fecal samples from AD patients and healthy volunteers by 16s rRNA sequencing. These fecal samples will subsequently be used to perform *in vitro* fermentation experiments to analyze the microbial metabolism of polyphenolic compounds. The resulting metabolites will be analyzed for potential differences using LC-MS/MS. The bioactivity of the metabolites will be assessed in AD specific *in vitro* models, including iPSC-derived APOE4/4 neurons and microglia, addressing several aspects of the disease pathology.

B.1.3 Layman's Summary

Alzheimer's Disease (AD) is a neurological disease associated with decreased cognitive function which is becoming an increasing societal concern because of our aging population. Scientists have not yet found a cure for AD, and even the exact

biological mechanism causing the disease has not been clarified. Potential medicines that have been developed failed in clinical trials, probably because they only target on a small aspect of the disease, while it is now recognized that AD is caused by multiple pathological mechanisms.

Compounds from nature that have long been considered to have beneficial health effects are polyphenols. These compounds can be found in a lot of plants and have a lot of different classes and subtypes, each of them carrying out slightly different effects on the body. They are mostly known for their antioxidative effect but are now also gaining attention for neurological conditions. For AD, specifically epigallocatechin-3-Gallate (EGCG), derived from green tea leaves, and resveratrol, present in red grapes, have been identified as potential drug candidates, as they target multiple pathological mechanisms.

Because these compounds are orally ingested, a main challenge is whether they can reach the brain. After ingestion, they are namely extensively processed by the bacteria present in the gut, altering the structure of the molecules. This, in turn, has an effect on their uptake and biological activity. What further complicates this, is that there is a strong connection between the gut bacteria and the brain, and patients with neurological conditions, such as AD and Parkinson's disease, have been shown to have different gut bacteria compositions compared to the healthy population. These differences in gut bacteria may alter polyphenol metabolism in such a way, that the beneficial effects of the molecules do not occur in AD patients who have an altered gut bacteria composition.

The goal of this study is therefore to study the link between altered gut bacteria composition in AD and the effect of the different metabolites on their disease-modifying properties. We will firstly identify differences in gut microbiome composition by collecting fecal samples from AD and healthy volunteers and analyze the bacteria based on their genetics. Once we extract EGCG and resveratrol from green tea and red grapes, we will use the fecal samples to metabolize the compounds and identify the resulting metabolites using chemical analytical techniques. We will study which metabolites can enter the bloodstream by mimicking the gut and blood brain barrier using a cell model and checking whether the molecules are transported by the cells from one compartment to the other. The metabolites will be tested for disease-modifying properties using several *in vitro* assays resembling AD. Specifically, we will look at their interaction with pathological protein aggregates, but we will also do tests on a cellular level. For this, we will use stem cells introduced with a specific mutation that is often present in patients with AD. In the lab, we can differentiate these AD stem cells into specific brain cells, neurons and microglia, and use them to run multiple experiments with our metabolites.

B.1.4 Keywords

AD, polyphenols, dysbiosis, microbiome, apoE4

B.2 Scientific Proposal

B.2.1 Research Topic

In this time and age where life expectancy is steadily increasing, the incidence and burden of age-associated neurodegenerative diseases are consequently becoming a growing societal challenge, with Alzheimer's Disease (AD) affecting over 40 million people worldwide (1). According to Alzheimer Nederland, dementia is the fastest growing cause of death in the Netherlands, and cases of dementia have explosively increased at a 6-fold rate from the 1950s until now. This has led to expenses of over 10 billion euros in 2020 for dementia-related care in the Netherlands, illustrating the serious societal impact of the disease (2). AD accounts for 60-80% of all cases of dementia and leads to debilitating syndromes manifesting in memory loss and severe cognitive dysfunction (3). For decades, AD research was focused on the outdated perception that the disease was merely caused by the accumulation of a particular amyloidogenic protein, amyloid-beta (Aβ), and abnormal tau aggregates in neurons. However, several Aβ and tau-clearing agents have been developed but were unsuccessful in showing any symptomatic relief in clinical trials (4). It is now widely recognized that AD is a complex, multifactorial disease and cannot solely be attributed to Aβ accumulation and tau-pathology (5). There is, therefore, a growing interest in the development of pleiotropic agents that target multiple pathways for the treatment of AD. Failure of high-cost medicinal products in clinical trials has led scientists to turn their focus towards potential therapeutics provided by nature, phytochemicals. (Poly)phenols are molecules derived from plants that have been long recognized for the great benefits they impose for general health (6). Specifically for AD, research has shown that (poly)phenols are able to interfere with multiple disease mechanisms (7)(8)(9). However, the remaining challenges for (poly)phenols as therapeutic candidates mainly relate to their bioavailability, as they are subject to metabolizations by gut microflora in order to be absorbed and exert their neuroprotective effect. However, gut dysbiosis is a common feature of neurological disease, and therefore bioavailability of dietary (poly)phenols might be altered in patients with AD (10).

Plants' potential in AD: (Poly)phenols and their neuroprotective actions

(Poly)phenols are a group of naturally occurring organic compounds that are found in many plant foods and have widespread health benefits as shown by epidemiological and clinical studies (11)(12). (Poly)phenol-rich diets and supplements containing extracts have shown to effectively reduce the risk and prevalence of a broad spectrum of conditions such as cardiovascular disease, diabetes, cancer, immune complications, and moreover exhibit anti-ageing effects (11–15). (Poly)phenols are primarily known for their strong antioxidative capacities as a result of their radical scavenging and metal chelation abilities. However, beyond their antioxidative function, recent evidence indicates that these agents might even exert

their effect by modulating cell signaling pathways (16). Regarding neurodegenerative disease, in particular Alzheimer's disease, these compounds are increasingly becoming of interest because of their ability to interfere with multiple aspects of the disease. Studies have shown their ability to interact with and disaggregate tau and Aβ- fibrils (17)(18), enhance the inhibitory potential of acetylcholinesterase (19), and modulate neuroinflammatory pathways through with NFkB signaling and complement activation as shown by transcriptomic data in microglia (20).

(Poly)phenols are structurally defined by containing at least one aromatic ring with one or more hydroxyl groups. These compounds among one of the most ubiquitous bioactive molecules in plants, with more than 8000 phenolic structures currently known (13). Most often, (poly)phenols exist as glycosides, conjugated to one or more sugar residues linked to a hydroxyl group or aromatic carbon. However, conjugations with many other organic molecules, such as amines and lipids, also naturally occur (13). Even though (poly)phenols exist in this tremendous chemical diversity, they are generally classified into different groups based on their number of phenol rings and the structural elements that binds these rings. The four groups resulting from this classification are phenolic acids, flavonoids, stilbenes, and lignans (13). The most abundant among these groups are the flavonoids, accounting for half of the naturally occurring (poly)phenols. These share a common structure containing two aromatic rings in the C6-C3-C6 general conformation. Flavonoids can be further divided into six subclasses based on the type of ring structure: flavonols, flavones, isoflavones, anthocyanidins, and flavanols (catechins and proanthocyanidins). Phenolic acids are another group of (poly)phenols, which are classified into hydroxyl benzoic and hydroxyl cinnamic acids based on their C1-C6 and C3-C6 backbones, respectively. Stilbenes consist of two phenol rings coupled by a methylene bridge. Finally, lignans are a class of compounds containing two phenolic moieties connected by the dimerization of two cinnamic acid residues (13) (Table 1).

Dietary (poly)phenols	**Flavonoids**	Flavanols	O, OH, O
		Flavones	O, O
		Isoflavones	O, O
	Phenolic acids	Hydrocinnamic acid	OH, O
		Hydroxybenzoic acid	O, OH

	Lignans	
	Stilbenes	

Table 1 | Polyphenol classification system and the general chemical structures of the different classes (21).

Two main (poly)phenolic compounds have been of interest as a potential therapy for AD. Epigallocatechin-3-Gallate (EGCG) belongs to the flavonol subgroup of (poly)phenols and is the most abundant catechin found in green tea, accounting for roughly 50-80% of catechins (13). In contrast to black tea, green tea is not fully fermented and therefore maintains the native structure of (poly)phenols. As shown by epidemiological studies, the consumption of green tea is associated with a lower risk of cognitive impairment (22). EGCG has extensively studied in binding studies and shown to effectively disaggregate amyloid and tau fibrils *in vitro* (18). Besides EGCG, the most studied (poly)phenol for AD therapy is resveratrol, a stilbene particularly present in red grapes, and therefore concentrated in red wine and grape juice. The mechanism by which resveratrol exerts its neuroprotective effect is thought to be similar to the effects of caloric restriction, which occurs through activation of SIRT1 (23). In a recent study screening of (poly)phenolic compounds in a herpes simplex virus type I (HSV-1) induced model of AD, green tea catechins and resveratrol showed to have the strongest neuroprotective effect and reduction of plaque formation, further supporting the potential of these agents in the treatment of AD (24). Resveratrol has been shown to be absorbed through passive diffusion or membrane transporters from the intestine into the bloodstream, where high levels of its metabolites can be detected. The parent molecule has been shown to cross the blood brain barrier (BBB), however only low levels can be detected in the brain in contrast to high levels of its metabolites found in the bloodstream (25)(21). Even though resveratrol as a parent molecule has been extensively studied, the bioavailability and bioactivity of its derivatives in the brain remain largely elusive. Studies into the bioavailability and bioactivity of polyphenol metabolites could lead to further understanding of how these compounds could exert their neuroprotective effects and lead to the identification of a potential phytochemical drug candidate.

Linking dysbiosis to bioavailability of polyphenolic compounds in AD

As stated before, the potential therapeutic effect of dietary (poly)phenols in AD is greatly dependent on their bioavailability in the bloodstream and, in turn, in the brain. The majority of dietary (poly)phenols exist as esters, glycosides or polymers and are therefore too structurally complex to be readily absorbed. After oral ingestion, only 5-10% of the total (poly)phenol intake is absorbed in the intestine, while the remaining 90-95% accumulates in the large intestinal lumen (26)(15). Here, the compounds are subject to hydrolyzation by intestinal enzymes or colonic microflora into relatively

simple low-molecular-weight metabolites, commonly referred to as phenolic acids, which are known to mediate widespread physiological effects (27)(10). Microbial enzyme transformations of complex (poly)phenols include C-ring cleavage, decarboxylation, dihydroxylation, and demethylation. However, different classes of polyphenols may be metabolized by different bacterial species, therefore differences in gut microbiome composition may account for differences in bioavailability between individuals (28).

Neurodegenerative disorders have been extensively linked to gut dysbiosis and considering that the gut microbiome accounts largely for (poly)phenol metabolism into bioavailable compounds, it is important to consider the differences in microbiome composition between AD patients and the healthy population (29)(30). A recent *in vivo* study even showed that the beneficial effect on cognitive impairment of dietary (poly)phenols was attenuated in mice with antibiotic-induced dysbiosis compared to controls. Antibiotic-treated mice showed altered plasma concentrations of bioactive (poly)phenol metabolites, highlighting the importance of the microbiome in the bioavailability and bioactivity of (poly)phenols (10). Differences in gut microbiome composition between AD patients and healthy volunteers have already been investigated, however the specific link between altered microbial species in AD and polyphenol metabolism remains to be studied (31). For resveratrol, researchers have shown that interindividual differences in microbiome composition was closely related to presence of specific metabolites. Specifically, they identified *Slackia equolifaciens* and *Adlercreutzia equolifaciens* as the primary responsible species for microbial resveratrol metabolism, which are known to be depleted in patients with AD (32)(33). Regarding EGCG, *Enterobacter aerogenes*, *Raoultella planticola*, *K. pneumoniae* subspecies *pneumoniae*, and *Bifidobacterium* longum subspecies *infantis* are known to hydrolyze EGCG into gallic acid and EGC (34). Of these, *Bifidobacterium* has been found to be decreased in the gut microbiome of AD patients (30).

From classical views on AD pathogenesis to complex disease mechanisms and novel research approaches

Pathology of AD is generally described by the excessive accumulation of Aβ plaques and intracellular neurofibrillary tangles (NFTs). Mechanistically, Aβ is formed through sequential hydrolysis of the transmembrane amyloid precursor protein (APP) by β-endoproteases resulting in the release of the sAPPβ ectodomain, followed by cleavage of the remaining 99 amino acid APP carboxy-terminal fragment (β-CTF) by γ-secretase. β-CTF can be cleaved at various sites, generating Aβ fragments of variable lengths. The major Aβ-species in the brain comprise 40 and 42 amino acid peptides, of which the hydrophobic Aβ42 has a stronger propensity to aggregate and is therefore considered the key factor in initiating plaque formation (35). In AD pathogenesis, Aβ fragments assemble into a variety of unstable oligomeric species, which further aggregate into short protofibrils that eventually polymerize into longer insoluble fibrillar assemblies (36). Particularly, oligomeric forms of Aβ have shown to exert toxic effects, such as N-methyl-D-aspartic acid receptor (NMDAR)-mediated excitotoxicity,

intracellular calcium imbalance, mitochondrial dysfunction, and production of ROS (37). NFTs are formed by hyperphosphorylated microtubule-associated tau protein. Physiologically, tau is a soluble protein promoting tubulin polymerization and thereby regulating stabilization and dynamics of microtubules. Tau proteins exist in six isoforms due to alternative splicing of exons 2, 3 and 10, which either contain three or four microtubule-binding domains, thereby named 3R and 4R isoforms. Upon phosphorylation by protein kinases, tau is released and promotes microtubule disassembly. Aberrant hyperphosphorylation of tau and subsequent misfolding ultimately lead to aggregates forming pathological inclusions (36). Parallel to the amyloid and tau cascade hypotheses, scientific evidence accumulated over the past four decades pointed towards dysfunctional cholinergic neurotransmission as a cause of AD-related cognitive impairment. This has eventually led to the development of cholinesterase inhibitor therapies for AD, however these therapies are primarily aimed at providing symptomatic relief rather than exerting disease-modifying effects (35). Despite the development of numerous rodent AD models through overexpression of amyloid and tau pathology-related genes, failure of anti-amyloid and tau agents in human clinical trials indicates that the mechanisms driving AD pathogenesis extend beyond these protein aggregates (4).

The ε4 variant of the apolipoprotein E gene (APOE4) has been established as the strongest genetic risk factor for the development of sporadic late-onset AD. Individuals carrying one allele of the APOE4 variant have 2-4 times increased risk of developing AD compared to individuals with two copies of the APOE3 allele and having two copies of APOE4 even increases the risk 8-12 times (38). Apolipoprotein E (apoE) is an important regulator of lipid homeostasis, mediating transport and absorption of dietary lipids from the peripheral circulation to the brain. The different isoforms of apoE influence their ability to bind lipids, receptors, and Aβ. However, the exact modulatory effect of apoE on AD pathogenesis is highly complex and remains largely unknown (39). More recently discovered single nucleotide polymorphisms (SNPs) associated with greater late-onset AD risk, however, reside in genes encoding proteins involved in immunomodulation, such as triggering receptor expressed on myeloid cells 2 (TREM2), phosphoinositide phospholipase Cγ2 (PLCG2) and CD33 (40). An excessive pro-inflammatory response is a becoming progressively acknowledged as a key feature of AD pathogenesis. Microglia are the resident immune cells of the brain and exert important neuroprotective functions under physiological conditions, as they continuously survey the microenvironment of the brain with their processes, sensing and responding to damage signals. Upon recognition of a pathological signal, microglia are able to internalize and degrade these pathogens (41). In AD brains, dysfunctional microglia might contribute to neurodegeneration due to sustained activity and secretion of pro-inflammatory cytokines, however the exact role of microglia in AD is highly complex and not yet fully elucidated (41). As revealed by transcriptomic mouse studies, under diseased conditions microglia gradually transition from homeostatic to disease-associated

microglia (DAMs) through upregulation and downregulation of an intricate network of genes (42).

Traditionally, AD was studied using rodent models overexpressing amyloid-related genes, such as APP and PSEN1. These approaches, however, are focused on early-onset familial AD, which only occurs in 1% of all cases of AD (38). Sporadic forms represent the vast majority of AD cases, while these sporadic forms are especially hard to study as the underlying cause and mechanisms are not yet fully understood. Ever since the emergence of induced pluripotent stem cells (iPSCs), several human-based models have been developed which have revolutionized preclinical research into AD. iPSC-lines have been generated from AD patients with familial as well as sporadic forms, and generation of these cells into neurons in 2D cultures revealed that both lines exhibit AD-related pathologies, such as higher levels of Aβ, phospho-tau, and endoplasmic reticulum (ER) stress (38). In order to better recapitulate disease pathologies and achieve higher model complexity, 3D neuronal *in vitro* models have been developed that show important AD hallmarks. Contrary to 2D models, amyloid plaques and NFTs are able to form within 3D neuronal structures. Nowadays, even more advanced iPSC-derived multicellular models, brain organoids, have been developed that allow examination of neuron-glia interactions in the 3D context. As a recent study on cerebral organoids showed, even microglia are present within cerebral organoids, opening new avenues to study inflammation in neurological disease (43). The challenges of these high complexity models, however, reside in their low reproducibility and low cost-effectiveness, which should be considered before turning to these models for preclinical experiments (44). To conclude, novel *in vitro* technologies using human stem cell-derived systems have revolutionized pharmacokinetic and pharmacodynamic studies. These platforms could provide the means to further elucidate mechanisms by which (poly)phenols exert their neuroprotective effect and help translate these compounds into a potential therapy. Therefore, we propose to investigate (poly)phenols and their metabolites under the conditions of AD-related dysbiosis using solely human-based *in vitro* technologies and assess their biological activity in state-of-the-art disease-specific AD models.

B.2.2 Approach

TASK 1: Characterizing gut microbiome composition of AD patients

As shown by previous studies, dysbiosis is common in patients with AD (30)(45)(46). In order to confirm differences in gut microbiome composition, we aim to analyze bacterial strains from fecal samples of AD patients and healthy controls, which will later be used for metabolization studies with (poly)phenols. Considering the effect diet and environmental exposure can have on gut microbiome composition, ideally data should be collected from unrelated household pairs (typically spouses). This paired design has shown to reduce the influence of geographic and environmental factors in a study into multiple sclerosis and microbiome associations, therefore it could potentially enhance the power of our microbiome study for AD (47).

16S rRNA analysis is a technique often used to assess enterotype characteristics of fecal samples and is based on the sequencing of the prokaryotic 16S rRNA gene. The 16S rRNA gene is approximately 1500 base pairs long and contains conserved regions as well as nine variable regions, which is why it is widely used as a phylogenic marker gene. Universal PCR primers can target the conserved regions, allowing for amplification of the gene in a wide range of microorganisms. Discrimination between specific microorganisms can be accomplished through sequencing of the variable regions (48). Using previous data and databases, a library can be constructed in order to sequence and quantify the genes of interest.

For processing of the samples, stool will be collected by participants in DNA stabilizer-containing tubes, which also function as a lysis buffer. Samples can be stored in a domestic fridge for approximately 3 days before processing in the lab. Once in the lab, the samples will be transferred to an anaerobic cabinet, homogenized at 20% w/v in PBS and sieved through filters, after which the aliquots can be stored at -80°C. For the extraction of DNA from stool, several kits are commercially available. Costea et al. compared different kits and protocols for the processing of human fecal samples in metagenomic studies and found that the G'NOME kit (BIO 101) showed the overall best extraction quality and reproducibility, therefore this method will be followed (49).

Library construction involves the preparation of genomic DNA to be sequenced within the samples. The steps of library preparation include amplification of genomic DNA by PCR, amplicon size confirmation by gel electrophoresis, DNA quantification and normalization, PCR cleaning, and finally determining the concentration by quantitative PCR. The Illumina MiSeq system can be used for 16S metagenomic sequencing library preparation and includes the amplification of the 460 base pair long variable V3 and V4 using the following primer sequences:

16S Amplicon PCR forward primer:
5'
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
16S Amplicon PCR reverse primer:
5'
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTA
ATCC

After completion of the Illumina MiSeq system protocol and loading of the samples in the MiSeq sequencing plate, data can be analyzed following the 16S Metagenomics workflow within the MiSeq Reporter software. This software classifies organisms from the V3 and V4 amplicon based on the Greengenes database and provides various options for analyzing sequencing data, classifying reads at multiple taxonomic levels.

TASK 2: Identifying bioavailable (poly)phenols metabolites

Because of their potential as shown by previous studies, we aim to extract the (poly)phenolic compounds from grape skin and green tea, which consist of resveratrol and EGCG, respectively. Extraction of (poly)phenolic compounds from these matrices is an important step for the isolation and identification of these agents and can have various efficiencies based on different methods. Important factors influencing the extraction efficiency are choice of extraction solvent, pH, extraction time, and temperature. For (poly)phenols, methanol is usually the most efficient extraction solvent, followed by ethanol and finally water. Regarding pH, it is generally required to use low pH in the extraction solvent in order to prevent oxidation of the phenolic compounds, which is why acidified methanol is suitable for this purpose. In order to improve extraction efficiency, extraction cycles can be elongated and repeated, however excessive extraction times could cause degradation of the final compound due to oxidation (50).

The resulting solvent extraction requires an additional step of clean-up prior to analysis in order to remove all non-phenolic substances. A suitable method this purification is SPE, in which the target compound is retained in a sorbent cartridge and followingly eluted in an organic solvent. The separation of molecules by a column relies on their difference in polarity. In reverse phase high performance liquid chromatography (RP-HPLC), the column material is nonpolar, meaning that nonpolar compounds from the sample will adhere better to the column when present in a more polar solvent. Using a gradient elution for separation allows for the optimization of solvent polarity for the extraction of (poly)phenols from, for example, sugars and lipids present in the original extract. For this aim, we will use a C18 silica column preconditioned with methanol and water, and gradient elution for the mobile phases will be optimized for each sample in eluents as described in Figure 1.

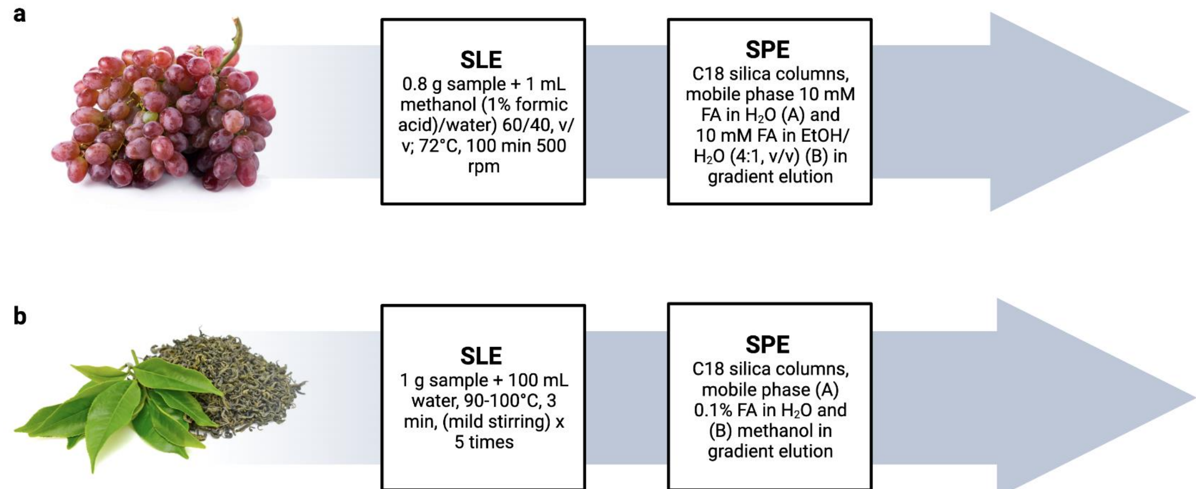

Figure 1 | Schematic workflow of polyphenol extraction from red grape skin **(a)** and green tea leaves **(b)**.

Based on our hypothesis and previous research stating that intestinal microflora play an important role in polyphenol metabolism, we will subject the extracted resveratrol and EGCG compounds to in vitro fermentation using fecal samples from AD patients and healthy volunteers. For this, commercially available Schaedler Broth will be inoculated with fecal samples (2% v/v) as this growth medium has been shown to best preserve the human gut microbiome complexity (ref. comparing different media in vitro fermentation). To mimic conditions in the distal large intestine, cultures should be maintained at pH 6.8 and a temperature of 37 °C under mild stirring in an anaerobic chamber (51). The batch cultures will be run for 48 hours, and medium will be collected at timepoints 0, 10, 24, and 48 hours.

Final identification and quantification of resveratrol and EGCG will be done as previously described through liquid chromatograph-tandem mass spectrometry (LC-MS/MS) in RP mode with C18 alkyl chains using a mobile phase of 0.1% aqueous FA and methanol as described in a previous study (52). The HPLC system will be coupled to a triple quadrupole MS connected by an electrospray ionization source (ESI) with positive ion mode selected as the ionization mode. Common metabolites of resveratrol are products of glucuronidation, sulfation, or hydrogenation, resulting in the metabolites as shown in Figure 2, which have a mass range between 214.260 and 404.37 g/mol. Therefore, these metabolites include dihydroreservatrol, trans-resveratrol-3-O-sulfate, and trans-resveratrol-3-O-glucorinide, which have a mass range between 110.11 and 292.28 g/mol. For EGCG, the intestinal microbial metabolism has been described by Zhao et al. and is illustrated in Figure 3.

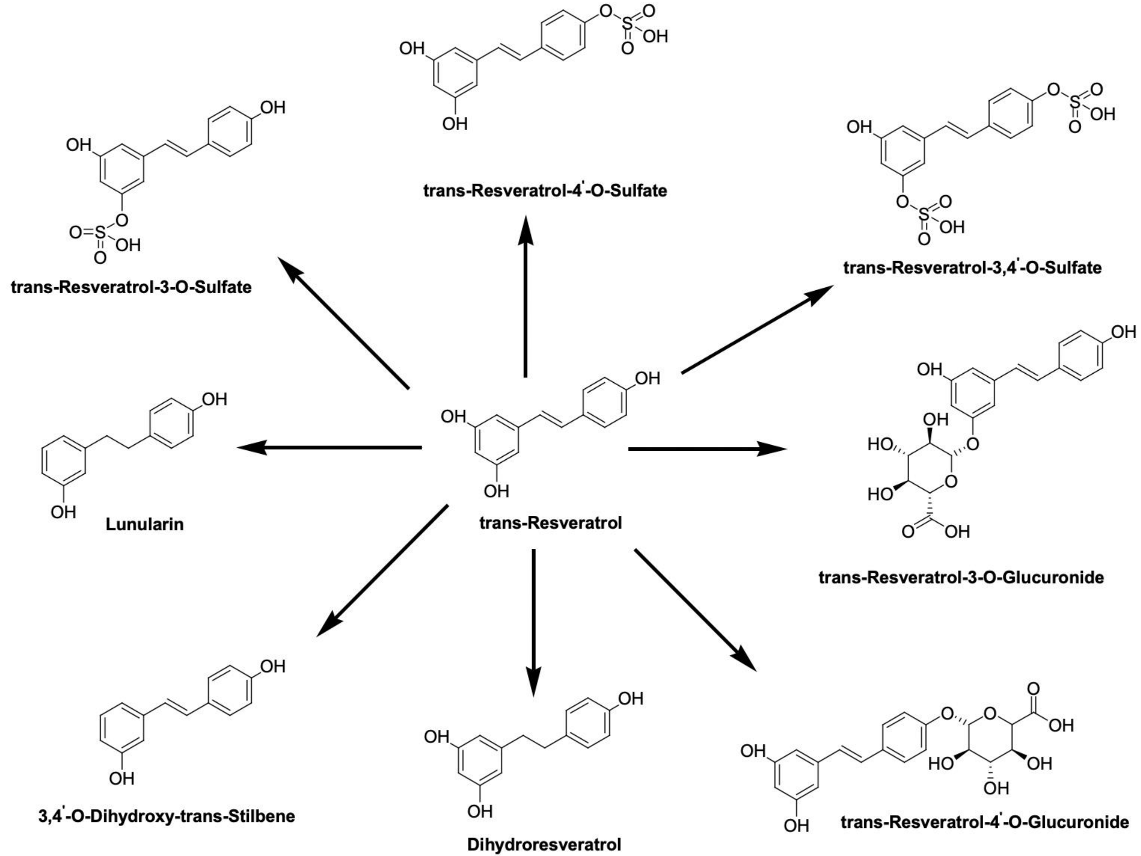

Figure 2 | Figure from the article by Saad et al. depicting common metabolites of resveratrol (53).

gallic acid
pyrogallol
pyrocatechol
EGCG
EGC
EC
4–HPAA
1-(3',4',5'-trihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)propan-2-ol
5-(3',4'-dihydroxyphenyl)-pentanoic acid
3,4-DHPPA
delta-(3',4',5'-trihydroxyphenyl)-gamma-valerolactone
1-(3'-hydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)propan-2-ol
5-(3',5'-dihydroxyphenyl)-pentanoic acid
3,5-DHPPA

Figure 3 | Figure from the article by Zhao et al. showing the microbial metabolites of (-)-epigallocatechin gallate (EGCG) (27).

Metabolization by gut bacteria could greatly impact the bioavailability of the polyphenolic compounds. We will employ a gut epithelial barrier *in vitro* model to examine the transport of polyphenol metabolites along the barrier and into the bloodstream. The intestinal epithelium consists of a monolayer of cells predominantly containing enterocytes (54). Considering cost-effectiveness, we will opt for a conventional 2D monolayer in a transwell model. The cells within this system will be polarized Caco-2 cells as they have been the gold standard for ADME studies and accepted by regulatory authorities (54). For bioavailability to the brain, compounds must be able to cross the BBB, which is composed of a single endothelial cell layer lined with brush-like glycoproteins (glycocalyx). The endothelial cells (ECs) are surrounded by a basal lamina extracellular matrix, covered by pericytes and astrocytic end feet (55) (Figure 4a). We will mimick the BBB *in vitro* by employing a transwell co-culture model of hiPSC-derived BBB ECs with commercially available human primary astrocytes and pericytes, in line with a previously characterized model by Appelt-Menzel et al. (56). Briefly, BBB ECs are generated from iPSCs by first culturing them for 6 days with an unconditioned medium. Thereafter, cells are cultured for 2 days in human Endothelial-SFM supplemented with 1 % platelet-poor plasma derived bovine serum, human fibroblast growth factor (FGF) and retinoic acid. Transwell inserts are coated with a mixture of collagen IV and fibronectin before plating the cells (56).

Monolayer integrity should be confirmed by monitoring transendothelial electrical resistance (TEER >100 $\Omega \times cm^2$). Further confirmation of cell identity and function will be done through analysis of EC markers (i.e., VE-cadherin, vWF, and Ulex), tight-junction proteins (i.e., claudin-5, occluding and ZO-1) and expression of influx transporters (i.e., Glut-1, SLC7A5, and CD220) and efflux transporters (i.e., P-pg, ABCG2 and MRP-1) (57). Metabolites identified by the previous fermentation experiment will be added to the apical compartment of the transwell systems and resulting bioavailable compounds will be identified in the basolateral compartment medium by LC-MS/MS as described earlier (Figure 4b).

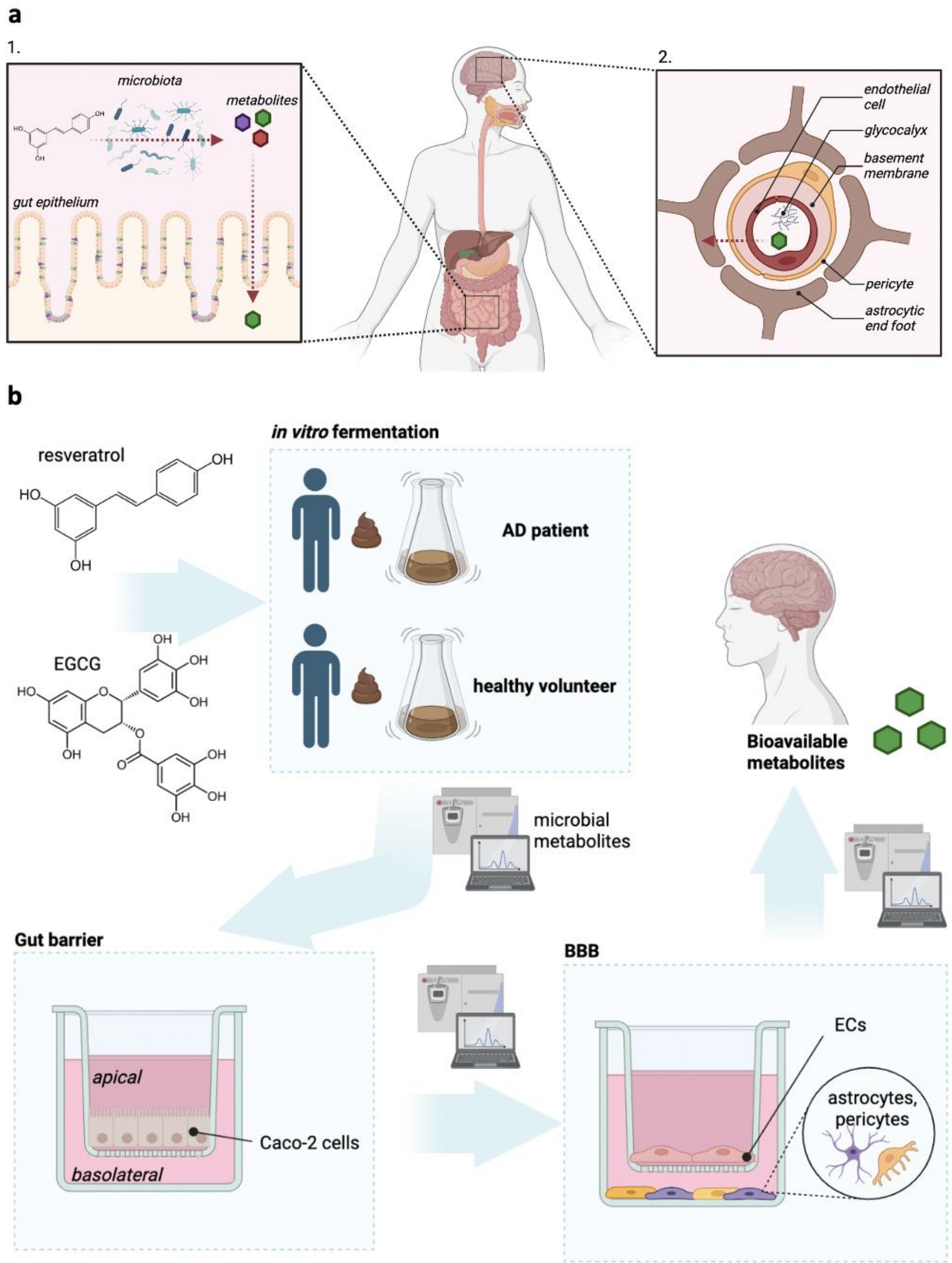

Figure 4 | (a) After oral ingestion of polyphenols, the compounds are subject to metabolizations by gut microbiota before they are absorbed through the gut intestinal epithelium (1) into the bloodstream. To exert their neuroprotective effect, they additionally have to be able to cross the BBB (2). **(b)** Schematic workflow for the identification of bioavailable metabolites. This comprises of subjecting the compounds to *in vitro* fermentation followed by analysis and isolation of the resulting metabolites. Followingly, the metabolites will be added to the apical compartment of a transwell containing Caco2 cells. The medium from the basolateral compartment will be analyzed

for compounds and checked for their ability to reach the brain using an *in vitro* BBB transwell co-culture model.

The concentrations of metabolites used for further bioactivity studies will be based on maximum plasma concentrations of the compounds as shown by previous studies and their uptake in the *in vitro* BBB model. For EGCG and metabolites, peak plasma concentrations vary between 77.9 ± 22.2 and 223.4 ± 35.2 ng/ml after administration of 20 mg green tea solid per kg bodyweight or 2 mg/kg body weight of pure EGCG dissolved in 200 ml water (58). For resveratrol, generally higher concentrations are required due to short half-life of the compound. In a study where the pharmacokinetic profile of 500 mg resveratrol doses was assessed, the peak plasma concentrations of metabolites ranged between 4,083.9±1,704.4 ng/ml and 1,516.0±639.0 ng/ml (59). Notably, these concentrations were well tolerated by the participants. The permeability coefficient (P_e) of each compound will be determined using the BBB *in vitro* as described previously (60). The cleared volume for each transport direction (apical-to-basolateral and basolateral-to-apical) should firstly be determined in order to find the efflux ratio (ER), which can be calculated by the following equations:

$$\text{Cleared volume}_{\text{ApicalToBasolateral}} = \frac{C_{\text{basolateral}} \times V_{\text{basolateral}}}{C_{\text{apical}}}$$

$$\text{Cleared volume}_{\text{BasolateralToApical}} = \frac{C_{\text{apical}} \times V_{\text{apical}}}{C_{\text{basolateral}}}$$

where we will use the concentrations of the metabolites (C) and the volumes (V) of either basolateral or apical side. The slope of the clearance curve for the BBB model (PS_{total}) and a cell-free insert control (PS_{insert}) are used to calculate the permeability multiplied by the area of the endothelial monolayer using the following equation:

$$\frac{1}{PS_e} = \frac{1}{PS_{\text{total}}} - \frac{1}{PS_{\text{insert}}}$$

The final P_e value is determined by dividing PS_e by the surface area of the insert.

TASK 3: Assessing disease-modifying properties of bioavailable (poly)phenol compounds on in vitro models of AD

In order to assess the bioactivity of polyphenolic microbial metabolites in AD patients versus healthy volunteers, we will run several *in vitro* experiments representing various aspects of AD. Therefore, we aim to study their biological effect on protein level, neuron level, and microglia level.

To explore the anti-amyloidogenic activity of the polyphenolic metabolites, we will assess the inhibitory effect of the compounds on aggregation and their destabilizing

activity on Aβ1-42 fibrils in line with previously described experiments by Choi et al. (61). Briefly, to study their inhibtiroy effect on Aβ1-42 aggregation, recombinant Aβ1-42 peptides will be incubated with various concentrations of the metabolites. Amyloid fibrils will be detected using thioflavin T (ThT), which will emit a fluorescent signal upon binding to fibrils. To assess the destabilizing effect on Aβ1-42 fibrils, recombinant Aβ1-42 peptides will firstly be incubated with ThT and allowed to form fibrils. Afterwards, different concentrations of the metabolites will be added to see if there will be a decrease in fluorescent intensity over time. To further confirm structural changes in the Aβ1-42 fibrils induced by polyphenol metabolites, we will perform SDS-PAGE electrophoresis and immunoblotting with an anti-Aβ antibody. The electrophoresis will separate the fibrils (250 kDa) into oligomers (8-30 kDa) and monomers (4 kDa) based on their molecular mass, and this will be done in denatured condition and after cross-linking to study both the inhibitory effect on aggregation and the disaggregating effect (Figure 5). In addition, the SDS-PAGE electrophoresis experiment can be used to analyze the assembly of Aβ into toxic oligomeric forms, which are also included in this study because of their unprecedented role in AD progression.

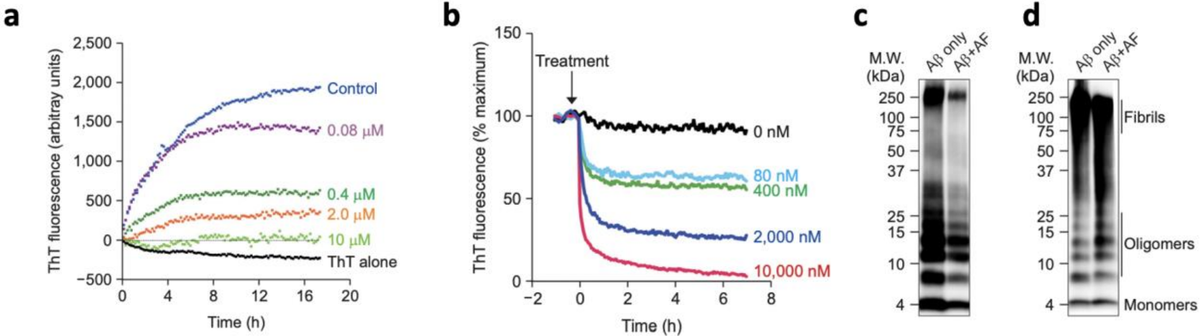

Figure 5 | Results by Choi et al. on their study of anti-amyloidogenic properties of polyphenolic bioflavonoids (61). **(a)** Aβ1-42 aggregation assay showing inhibitory effects of various amentoflavone (AF) concentrations through fluorescent intensity of ThT over time. **(b)** Aβ1-42 fibril disaggregation as measured by ThT fluorescence over time in various AF concentrations. **(c-d)** SDS-PAGE electrophoresis with Aβ immunoblotting showing aggregation inhibition in denatured condition **(c)** and destabilizing capacity after cross-linking **(d)**.

AD cannot solely be attributed to protein aggregate formation; therefore cellular assays are required to mimic the complexity of the disease more accurately. Recent research has shown that isogenic apoE4 iPSC-derived brain cells exhibit several molecular and functional abnormalities recapitulating an AD phenotype, which are listed in Table 2. Based on these results and taking into consideration that sporadic forms of AD represent the vast majority of the cases, we will design our bioactivity studies using APOE4/4 iPSC-derived neurons and microglia.

Cell type	Pathological feature
Neuron	• Increased $A\beta_{42}$ secretion (62) (63) • Elevated synaptic activity (62) • Increased number and size of early endosomes (62) • Prone to degeneration (63) (64) • Intracellular ApoE4 accumulation (63) • Elevated p-tau levels (63)
Microglia	• Amoeboid-like morphology; fewer and shorter processes (62) • Downregulation of genes associated with cell movement and development, upregulation of genes involved with immune response (62) • Reduced phagocytic activity (62) (65) • Aggravated inflammatory response (65) • Impaired metabolism (65)

Table 2 | Molecular and functional features in iPSC-derived brain cells with the APOE4/4 genotype that relate to AD pathology

Isogenic iPSC lines from a healthy donor will be generated containing the APOE4/4 for genotype for AD and APOE3/3 genotype as a control, as this is considered the neutral and most common isoform (66). The APOE4/4 model for AD and APOE3/3 control model will be used for all the cell experiments described below. The difference between the apoE4 and apoE3 isoforms resides in position 112, where apoE4 contains an arginine whereas apoE3 has a cysteine at this position. Using the protocol described by Lin et al., isogenic lines can be generated by converting APOE3 to APOE4 (62). As described in this study, a CRISPR/CAS9-APOE single guide RNA (sgRNA) with the 5′-CCTCGCCGCGGTACTGCACC-3′ sequence will target amino acid 112 within 10 nucleotides from the site to anneal the following oligomer pairs:

5′- CACCGCCTCGCCGCGGTACTGCACC-3′
5′-AAACGGTGCAGTACCGCGGCGAGGC-3′

These pairs are cloned into the pSpCas9-2A-GFP plasmid (Addgene #48138). Single-stranded oligodeoxynucleotides (ssODNs) are used as donor templates for efficient insertion of the DNA fragments. The ssODNs contain a silent mutation at the protospacer adjacent motif (PAM) to prevent recurrent Cas9 editing (62). The sequence used to convert APOE3 to APOE4 is as follows:

GAGGAGACGCGGGCACGGCTGTCCAAGGAGCTGCAGGCGGCGCAGGCCCG
GCTGGGCGCGGACATGGAGGACGTGCGCGGCCGGCTGGTGCAGTACCGCGG
CGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAGGAGCTGCGGGTGCGCCT
CGCCTCCCACCTGCGCAAGCTGCGTAAG

For delivery of the CRISPR/Cas9 sgRNA and donor templates, iPSCs will be subject to electroporation using Nucleofector – Amaxa and Human Stem Cell Nucleofector Kit 1 (Lonza) as described previously (62). After electroporation, fluorescence-activated cell sorting (FACS) can be used to isolate successfully gene-edited cells based on their GFP expression.

Differentiation of iPSCs into induced neurons (iNeurons) will be performed according to the protocol as described by Zhang et al. (67). Briefly, lentivirus with rtTA and Ngn2-GFP expression will be produced in HEK293T cells by cotransfection of helper plasmids pRSV-REV, pMDLg/pRRE and vesicular stomatitis virus G protein expression vector. After single cell seeding of the iPSCs onto Matrigel-coated plates, the lentivirus will be added to the culture in mTeSR1 medium containing polybrene. The first two days, the cells will be cultured in N2/DMEMF12/NEAA medium containing human BDNF, NT-3, and mouse laminin. On day 2, the medium will be switched to Neurobasal supplemented with B27/Glutamax containing BDNF and NT-3 (67). According to the authors, iPSCs should be converted into functional neurons in less than two weeks. We will carry out characterizations at day 14 to ensure full maturation.

At day 14, differentiation of iNeurons will be confirmed through several molecular and functional characterization experiments as previously described with some minor adjustments (67). Briefly, cells will be stained for neuronal markers MAP2, synapsin, and β3-tubulin and expression quantified using image analysis software. Using qPCR, mRNA levels of neuronal markers can additionally be quantified, which could be Ngn2, NeuN, MAP2, BRN2, FOXG1. Using a Ca^{2+} indicator, Fluo-4-AM, Ca^{2+} transients within the iNeuron culture can be monitored through live cell imaging, indicating the presence of functional cells.

As shown in previous studies, APOE4/4 iNeurons can be used to study p-tau formation and $A\beta_{42}$ secretion (62)(63). In this study, the authors showed that p-tau was increased in APOE4/4 iNeurons compared to APOE3/3 neurons through Western blot analysis of p-tau-specific antibodies AT8, AT180, PHF1, and AT270 and by double immunostaining for p-tau (AT8/PHF1) and MAP2 for neurons (Figure 6). In the same study, this effect was ameliorated by the addition of a small-molecule corrector that renders apoE4 apoE3-like (63). We aim to examine this effect using the same type of experiments applied to the microbial polyphenol metabolites and investigate their potential ameliorating capacities on p-tau formation. Regarding Aβ, researchers have shown that APOE4/4 iNeurons exhibit a 20% increased secretion compared to APOE3/3 iNeurons (62). Using an ELISA assay, we can additionally study the effect of polyphenolic metabolites on $A\beta_{42}$ secretion in APOE4/4 iNeurons.

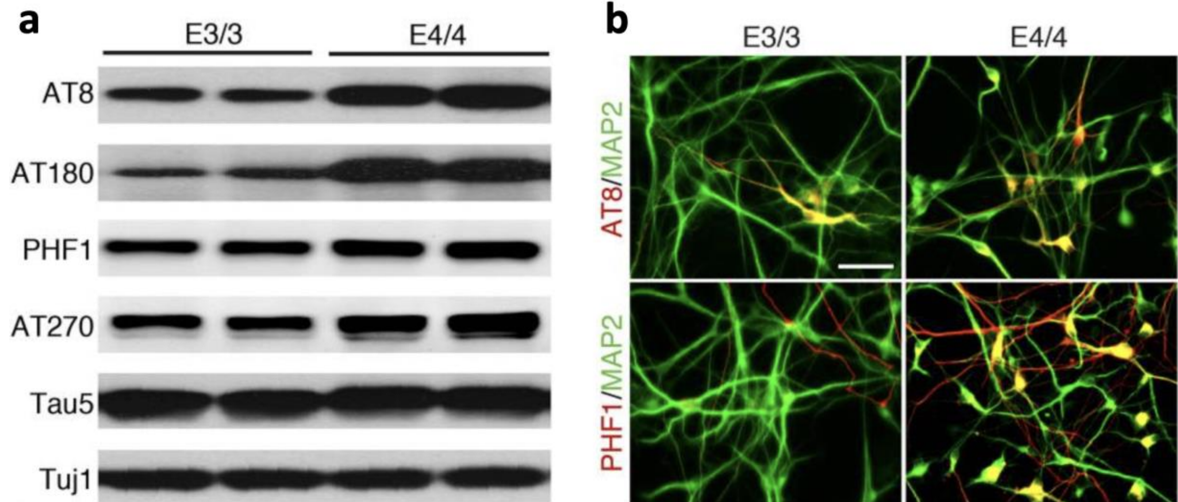

Figure 6 | Results from the study by Wang et al. showing increased p-tau in human APOE4/4 iNeurons compared to APOE3/3 through Western blot analysis **(a)** and immunostaining of p-tau (AT8/PHF1) and MAP2 (63).

A functional feature that can be studied using APOE4/4 iNeurons is hyperexcitability. Studies have shown that hyperactivity of neurons occurs in patients with both sporadic and familial AD even before pronounced cell loss occurs (68). This hyperexcitability can be observed in APOE4/4 iNeurons as increased miniature excitatory postsynaptic current (mEPSC) frequencies, which can be recorded in line with the electrophysiology protocol described by Lin et al. (62). In support of this experiment, Balez et al. showed that hyperexcitability in AD iNeurons could be moderated by the polyphenol apigenin (69).

In order to generate induced microglia (iMicroglia) from iPSCs, we will follow the recently published protocol by Chen et al. (70). In summary, after transfection with lentivirus containing transcription factors SPI1 and its cofactor CCAAT/enhancer-binding protein α, the cells are cultured in DMEM/F12 supplemented with N2 and NEAA, BMP4, bFGF, and activin-A. After one day, the medium is replaced with DMEM/F12/N2/NEAA containing human VEGF, SCF, and FGF2. From day 2 onwards, the cells will be cultured in DMEM/F12/N2/NEAA supplemented with human IL-34, M-CSF, and TGF-B1 (70). Authors state the cells differentiate into microglia-like cells within one week, therefore we will carry out characterizations at both day 7 and day 14.

Confirmation of microglial identity will be done through analysis of various markers. Microglial surface markers CD11b, TREM2, and CX3CR1 can be detected using flow cytometry, and additionally TMEM119, C1QA, GPR34 and CD11b expression will be analyzed using qPCR. Expression of stem cell marker POU5F1 can additionally be examined in order to confirm differentiation of the cells into a mature phenotype. Typical microglial markers, such as TREM2 and IBA1 will moreover be analyzed using immunostaining and quantified using image software analysis. To confirm

functional maturation of the induced microglia, several assays examining their inflammatory response, phagocytic capacity, nucleotide-evoked calcium signaling, and ability to migrate will be performed (70).

Multiple lines of research indicate that a sustained microglial inflammatory response through exposure to Aβ exacerbates AD progression. Research into APOE4/4 iMicroglia also show an aggravated inflammatory response in response to these stress stressors, therefore we want to incorporate this phenomenon in our bioactivity studies (65). A similar study has already been performed to test polyphenolic extracts from *Arabidopsis thaliana*, where the authors treated glial cells from APOE4-mutant mice with these extracts before exposing them to LPS or oligomerized Aβ (71). These extracts were shown to significantly attenuate the Aβ-induced inflammatory response based on their TNFα secretion as measured by ELISA. We will apply the same experiment to assess the immunomodulatory capacity of our extracted microbial metabolites, however we will use our human based APOE4/4 iMicroglia cell model.

An important function of microglia that is disrupted in AD and might contribute to further disease progression, is the uptake of Aβ. The uptake of Aβ by iMicroglia can be analyzed through ELISA and imaging experiments (62). For ELISA studies, iMicroglia will be incubated with oligomeric $A\beta_{42}$ for 24 hours and final $A\beta_{42}$ concentrations in the media will be determined using an $A\beta_{42}$ ELISA kit. These concentrations will be indicative of the fraction of $A\beta_{42}$ that has been taken up by the cells. To image $A\beta_{42}$ uptake by the cells, a fluorescent labeled $A\beta_{42}$ peptide can be purchased and used to treat the cells. Using live-cell imaging, uptake can be monitored in real time which allows for better examination of uptake efficiency.

Neuronal death as a result from sustained glial activation and secretion of inflammatory molecules has been considered a hallmark for AD (41). To investigate the protective capacity of the polyphenolic compounds against inflammation-induced degeneration, we will expose APOE4/4 iNeurons to various inflammatory stressors with or without polyphenol treatment. We will design these experiments in line with a previously published study by Balez et al., where the authors showed a neuroprotective effect of polyphenolic apigenin in iPSC-derived familial and sporadic AD neurons (69). Firstly, we will induce oxidative stress to iNeurons with and without polyphenol treatment to see if the metabolites protect neurons from degeneration. Followingly, we will use conditioned medium from APOE4/4 iMicroglia treated with and without polyphenol metabolites to assess the potential rescuing effect on neurodegeneration. In case the APOE4/4 iMicroglia conditioned medium do not induce neuronal degeneration, inflammatory iMicroglia can be generated by treating the cells with LPS and IFN-γ (69).

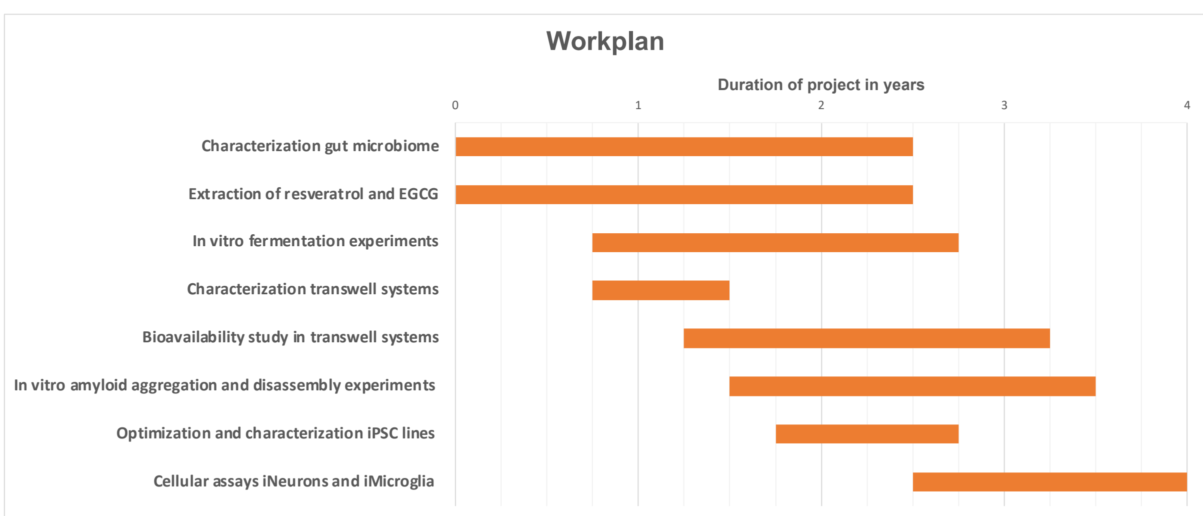

Figure 7 | Workplan for the tasks to be completed, taken into consideration their estimated duration and chronological order of the objectives.

B.2.3 Feasibility/Risk Assessment

The proposed project is based on long standing knowledge on beneficial effects of polyphenols combined with more recent insights in gut dysbiosis and AD. In every task, we support our experiments with previously published data, indicating the feasibility of our approach and the probability of our experiments leading to certain insights. In task 1, we were inspired by previous research providing a link between AD and dysbiosis. Therefore, we expect to also encounter differences in microbiome composition in our samples. As polyphenols are so heavily metabolized by the microbiome, we hypothesize that this will have a significant impact on polyphenol metabolism and therefore we expect to observe different metabolites resulting from different gut microbiome compositions. Lastly, the model we propose for our *in vitro* studies has been recently characterized to show AD-related phenotypical traits. iPSC-derived cells have even already been used to study the effect of other polyphenolic compounds, supporting the suitability of the chosen model for our proposal.

Nevertheless, possible setbacks are foreseen. If fecal samples from our volunteers do not show significant differences in gut microbiome composition, we will turn to existing literature and identify specific microbial species that have already been identified to be different in AD patients. These specific strains can subsequently be used to carry out the metabolization experiments of EGCG and resveratrol. Another possible challenge in this project might be the analysis of metabolites. Polyphenolic compounds can undergo extensive metabolizations into complex molecules. However, we will use previous literature describing these metabolization routes to limit our search for the correct molecules in MS data. In task 3, we use iPSC models to perform our bioactivity studies, which can be tedious to culture. We will follow previously published protocols on the differentiation of iPSC into our desired cell types. However, if any issues might arise, we will perform optimization

experiments to find the most suitable culture conditions, e.g., basal medium composition, supplementation, or cell culture substrate.

The techniques described in this proposal do not possess significant risks. Noteworthy, iPSC models require working with virus, in this case lentivirus. This, evidently, requires careful handling in line with good laboratory practice. This includes soaking any equipment in 10% bleach before discarding into biohazardous waste.

B.2.4 Scientific (a) and Societal (b) impact

Many research efforts have been focused at finding a treatment for AD but have failed in clinical trials (4). These included medicines based on very complex and advanced technologies, in which a lot of time and money has been invested. In our proposal, we aim to make a scientific impact (a) by showing the potential of compounds present in nature. Thereby, we hope to potentially inspire other researchers to shift focus on promising drug candidates in food and plants around us, instead of proceeding to make big investments to generate the most advanced medical product.

By studying gut microbiome composition in AD patients, we hopefully can show that neurological diseases have a strong association with the complex ecosystem present in our gut and should be considered as a system of pathological processes, rather than being attributed to a single phenomenon in one organ. If we prove that an altered gut microbiome composition affects the bioactivity of our compounds, this might also be the case for many other potential drug candidates. These results could stimulate further research into, for example, combination therapies, where the dysbiosis in the gut is targeted together with agents that might have a neuroprotective function.

Another aspect of this study that could have a scientific as well as a societal impact (b), is that we incorporate solely human based systems in our study design and exclude the use of animals. By using these models, we could inspire other researchers to also turn to *in vitro* study designs for their preclinical experiments. Efforts have already been made to optimize and characterize *in vitro* models for AD, however applied studies with these models are still very limited. We could provide further evidence that *in vitro* models are suitable systems for testing potential drug compounds. Moreover, these human-based models might provide insights that do not arise from animal models, as animal-derived cells are genetically so different from human cells.

B.2.5 Ethical considerations

No major ethical concerns are expected to arise during the course of this project. However, when working with patient material, extra care should be taken to assure complete anonymity. Because genetic data will only be taken from bacterial cells using 16S rRNA sequencing, the anonymity will be harbored and participants non-

identifiable. Moreover, informed consent should be obtained from the participants. This means that the participants should be adequately informed about the research and receive documents of disclosure containing the necessary information as described by the Dutch organization Central Committee of Research Involving Human Subjects (CCMO).

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