universität wien

DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

„Autophagy and Neuroinflammation in Alzheimer's Disease: A Nutraceutical Approach“

verfasst von / submitted by

Reinhard Gründler

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Magister der Pharmazie (Mag.pharm.)

Wien, 2020 / Vienna, 2020

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 449
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Diplomstudium Pharmazie
Betreut von / Supervisor:	Doz. Dr. Alexander Haslberger

I. Abstract

Aging and the emergence of age-associated illnesses are one of the major challenges of present society. Alzheimer’s disease (AD) is closely associated with aging and is defined by increasing memory loss and severe dementia. Currently, there are no therapy options available that halt AD progression. This work investigates three hallmarks of the disease (autophagy, neuroinflammation and senescence) and systematically analyzes if there is a beneficial effect from three substances derived from food sources, the so called „nutraceuticals“ epigallocatechin gallate, fisetin and spermidine on these hallmarks. To sum up, the results in this paper imply a positive outlook for the reviewed substances to qualify as a novel treatment option for AD in the future. Especially a combination of substances, targeting multiple hallmarks, could prove beneficial and warrants further exploration in clinical trials.

II. Zusammenfassung

Das Altern und die damit zusammenhängende Entstehung von altersbedingten Krankheiten sind eine der größten Herausforderungen der heutigen Gesellschaft. Die Alzheimer-Demenz (AD), für die es gegenwärtig keine ursächlichen Therapieoptionen gibt, steht dabei in engem Zusammenhang mit Alterungsprozessen und ist durch zunehmenden Gedächtnisverlust gekennzeichnet. In dieser Arbeit werden drei auslösende Faktoren der Krankheit (Autophagie, Neuroinflammation und Seneszenz) untersucht und systematisch analysiert, ob die drei von Nahrungsmitteln abgeleitete Substanzen, die so genannten "Nutraceuticals" Epigallocatechingallat, Fisetin und Spermidin eine vorteilhafte Wirkung auf diese Faktoren haben. Zusammenfassend zeigt die systematische Literaturrecherche, dass die untersuchten Substanzen in Zukunft eine vielversprechende Behandlungsoption für AD bilden könnten. Insbesondere eine Kombination von Substanzen die auf mehrere Faktoren abzielt, könnte sich als vorteilhaft erweisen und rechtfertigt weitere Untersuchungen in klinischen Studien.

III. Acknowledgements

First and foremost, I want to thank my supervisor, Dr. Alexander Haslberger, who has supported and advised me throughout the whole process of development and writing of this paper. Also, without him it would not have been possible to publish an excerpt of this work in a renowned scientific journal, for which I am very thankful.

Second, I want to thank my dearest advisor and supporter, Dr. Ulrike Haller. Without her continuous encouragement, motivation and never-ending universal support, I would have never been able to come to this point.

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V. Abbreviations

AD	Alzheimer’s Disease
AMPK	5' adenosine monophosphate-activated protein kinase
Aβ	beta-Amyloid
BBB	Blood-brain barrier
BCL	B-cell lymphoma
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
COX	Cycloogygenase
EGCG	Epigallocatechin gallate
FDA	U.S. Food and Drug Administration
GTCs	Green tea catechins
HEK-cells	Human embryonic kidney cells
IL	Interleukin
LoE	Level of evidence
NOS, NO	Reactive nitrogen species
NSAID	Nonsteroidal anti-inflammatory drug
RCT	Randomised controlled trial
ROS	Reactive oxygen species
SASP	Senescence-associated secretory phenotype
SCAP	Senescence-associated anti-apoptotic pathway
SR	Systematic review
TGF-β	Transforming growth factor beta
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor alpha
WHO	World Health Organization

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1 Introduction

1.1 Alzheimer's Disease – definiton and epidemiology

Alzheimer's Disease (AD) is an age-related, chronic neurodegenerative disease which causes 60-70% of all dementia cases.[1] Worldwide, an estimated 40 million people – mostly older than 60 years – have dementia, and until at least 2050, this number is said to double at least every 20 years[2]. About 6% of people over 65 years suffer from AD[1], which is defined by gradually worsening symptoms, but also includes a long, symptom-less pre-dementia stage, where the underlying pathogenetic processes start to form, often 10-20 years prior to first dementia symptoms.[3]

1.2 Pathogenesis and therapy approaches

Even after more than 35 years since the definition of AD[4], the definitive cause and pathogenesis of the disease are still not entirely known. Amyloid β (Aβ)-plaques and neurofibrillary tangles formed by tau protein aggregates have been discovered and linked to the disease. They have proven to be a good source for the exploration of molecular pathogenetic events, but targeting these hallmarks alone has not shown satisfactory results in finding a curative or disease-modifying treatment. Since then, many drug candidates have failed in clinical development - and since 2003, no new AD drug candidates have been approved by the FDA at all.[4–7]

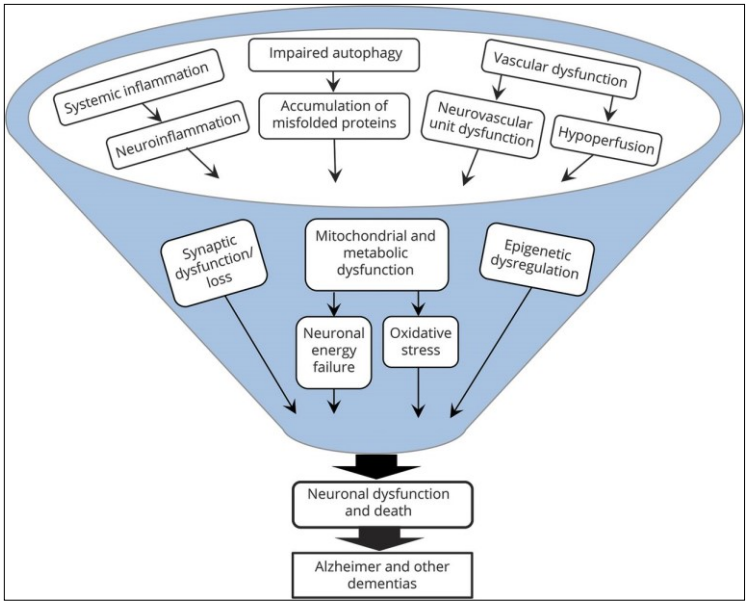

Figure 1: Age-related changes in biological processes that contribute AD formation. Edited after Hara *et al.* (2019)[6]

Regarding current therapy options, battling symptoms caused by the pathogenetic changes of Aβ-plaques and tau tangles also is state-of-the art, which limits therapy to relatively late-stages of the disease, in which patients already suffer from dementia symptoms. Apart from supportive care from family and other caregivers, drug therapy options mostly depend on four substances: cholinesterase inhibitors donepezil, rivastigmine, and galantamine, and glutamate antagonist memantine.[4] All of the above mentioned substances treat dementia in terms of memory loss and associated symptoms, but fail in slowing down or stopping the progress of the underlying mechanisms of AD. Therefore, it is in question if Aβ-plaques and neurofibrillary tangles alone are a sufficient targets in treating AD.

Several large phase III trials of anti-amyloid approaches in patients with mild to moderate AD have been published but the disappointing results contributed to a paradigm shift in the scientific community: [1,6] Many age-related changes in cellular and other physiological processes are now known that induce or contribute to the formation of Alzheimer's disease and other neurodegenerative diseases. It is now understood that the many different involved mechanisms and pathogenetic hallmarks (Fig. 1) demand the investigation of more diverse drug targets and definition of reliable biomarkers, aiming at the long pre-dementia stage of the disease at best. Prolonging the symptom-less phase and preventing the advancement of AD should be the main focus in concepting new therapies.

1.3 Goals and Motivation

Taking into account available therapy options, novel approaches in the prevention of AD and other neurodegenerative diseases could be necessary. One possible solution could be a combination of easily and cheaply available, food-derived substances (so called „nutraceuticals“) with a low profile of adverse reactions, aiming at the multiple involved characteristics of the disease. This work aims to explore a few potential candidates, categorize the available evidence and give recommendation for the clinical potential of those substances.

AD is defined by more than 9 pathogenetic hallmarks, most of which are age related: [6,8]

- Aβ-plaques
- tau proteins
- **impairment in autophagy and clearance of misfolded proteins**
- **neuroinflammation**
- **cellular senescence**
- mitochondrial and metabolic dysfunction
- vascular dysfunction
- epigenetic changes
- synaptic loss and dysfunction

This work will emphasize three hallmarks (marked in bold text above) and analyze if there is a beneficial effect from three substances derived from food sources (**Epigallocatechin gallate** (EGCG), **Fisetin** (F) and **Spermidine** (S)) on these hallmarks.

These substances were chosen because they are relatively easily accessible through food sources, and there is already some level of scientific evidence and findings regarding their effect on neurodegenerative diseases, that justifies further exploration of these potential candidates.

2 Material and Methods

The goal of the author was to conduct a systematic, comprehensive literature search of the discussed topics and to create an evidence-based overview if the data regarding discussed substances justifies a clinical reccomendation as potential drug candidates, hereby focussing on recent scientific developments. This was accomplished by systematically reviewing the available scientific evidence, literature and findings from clinical trial records. Search was conducted in multible databases, such as the scientific online network of the University of Vienna[a], Pubmed[b], the U.S. National Library of Medicine,[c] the Mendeley catalog of academic literature[d] among others. The following search terms were used: Alzheimer, neurodegenerative disease, autophagy, neuroinflammation, senescence, blood brain barrier, EGCG, polyphenol, fisetin, spermidine, nutraceutical and several other complementary terms.

Whenever evidence is cited in this paper that could suggest a potential impact of a substance on human health as a whole or AD in particular, level of evidence is rates and marked as follows:

Clinical evidence is rated following recommendations of the American Medical Association[9] and the Oxford Centre for Evidence-Based Medicine[10], in levels of evidence rated from 1a to 5 (see Table 1):

[a] https://usearch.uaccess.univie.ac.at/

[b] https://pubmed.ncbi.nlm.nih.gov/

[c] https://clinicaltrials.gov/

[d] https://mendeley.com

Level of Evidence	Type of Study
1a	Systematic review (SR) of randomised controlled trials (RCTs) and of prospective cohort studies
1b	Individual RCT with narrow confidence interval, prospective cohort study with good followup
1c	All or none studies, all or none case series
2a	SR (with homogeneity) of cohort studies
2b	Individual cohort study
2c	Outcomes research, ecological studies
3a	SR of case control studies, SR of 3b and better studies
3b	Individual case control study, nonconsecutive cohort study
4	Case series/case report, poor quality cohort studies
5	Expert opinion, bench research

Table 1: Levels of clinical evidence. Edited after the Oxford Centre for Evidence-Based Medicine's 2009 levels of evidence[9,10]

Non-clinical evidence such as *in vitro* studies or *in vivo* studies on animals is also marked as such. For example, if a study was carried out on cells *in vitro*, in this worked it is marked as: [LoE: *in vitro*]. If the level of evidence (LoE) e.g. corresponds to a multicenter RCT, it is marked as [LoE: *1b*]. Ex vivo studies are also marked as *in vitro.*

Clinical evidence has the highest level of impact and is best suited to recommend further pharmacological exploration of a mentioned substance. Nevertheless, because of the exploratorive nature of this work, often only non-clinical evidence was available. The guiding principle here was, that *in vitro* and *in vivo* studies can still, depending on their study design, serve as signals of potential beneficial or

harmful effects in humans. Following the National Research Council's *Framework for Evaluating Safety of Dietary Supplements*[11] validated *in vitro* studies can also stand alone as indicators of risk or benefit to human health. Data from non-validated *in vitro* studies can serve as hypothesis generators and as indicators of possible mechanisms of harm or benefit. In this context, *in vitro* assays are considered to be validated when their results are proven to predict a specific effect in animals or humans with reasonable certainty.[11]

3 Pathogenetic Hallmarks

3.1 Autophagy

Autophagy (also called autophagocytosis) describes the cellular process of clearance and recycling of damaged or aggregated molecules and cell structures, e.g. proteins, lipids or cell organelles. Also, it is a cellular form of survival technique, that a cell uses if it enters a state of nutrient starvation. The activity of autophagy decreases in aging model organisms, and stopping this decline has been associated with an increased lifespan in those organisms.[6]

3.1.1 Types

From a molecular biologic viewpoint, there are four main pathways that induce autophagy. In this chapter the pathways macroautophagy, microautophagy, chaperone-mediated autophagy, and others are descibed.

3.1.1.1 Macroautophagy

Macroautophagy is the most common type of autophagy, and the most well studied. It is a non-selective proteolytic process in eukaryotes, that clears the cell from damaged cell organelles and long-lived proteins, which are then catabolized. The first stage – nucleation - starts with the formation of an isolation membrane (*phagophore*), which elongates and forms a debris-enclosing body, called *autophagosome*. The autophagosome either directly fuses with a *lysosome*, or it initially fuses with late endosomes to form *amphisomes* - which later fuse with lysosomes. Then, lysosomal acidic hydrolases break down and degrade constituents into amino acids, free fatty acids and nucleic acids (Fig. 2).[12,13,14]

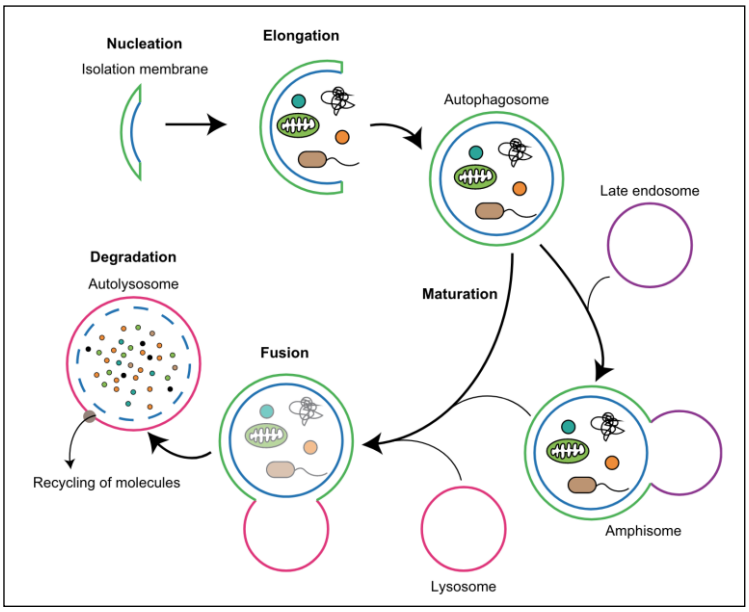

Figure 2: Schematic process of macroautophagy. Edited after Nakamura *et al.* (2017)[14]

In recent years, the understanding of macroautophagy and related processes has been amplified with the exploration of several genes that play distinct roles in the process. Also, a class of non-coding microRNA, so called miRNA, has been linked to play a role in the crosstalk between autophagy and apoptosis that potentially work as molecular switches between these two intimately connected processes and contribute to the cell fate decision.[15]

3.1.1.2 Microautophagy

Microautophagy differs from macroautophagy that the cell debris is enclosed directly in the lysosome and it does not require autophagosome formation for the delivery of targeted cargos to lysosomes. This is achieved by inward folding of the lysosomal membrane, a process called *cellular protrusion*. The underlying processes are not fully understood and until now, it is not known if microautophagy is selective or non-selective in targeting organelles to the lysosome.[12,16]

3.1.1.3 Chaperone-Mediated Autophagy (CMA)

CMA also does not require formation of an autophagosome. The activating pathways also are not fully known, but it is commonly understood as a selective mechanism. Kobayashi (2015) describes it as follows:

> *"With cooperation of the cytosolic chaperone Hsc70, other co-chaperones and the lysosomal receptor LAMP2A, proteins are delivered directly to the lysosomal lumen and degraded for intracellular quality control. (…) Although the effect of CMA on those proteins is limited, it is suggested that the impairment of CMA is tightly linked to the pathogenesis of neurodegenerative diseases and cancer, indicating that CMA is an indispensable pathway in maintaining health."* [12]

3.1.1.4 Other types

Other types of autophagy include *mitophagy* (selective degradation of mitochondria using autophagous methods) and *lipophagy* (degradation of lipids). Still, there is ongoing debate in the scientific community whether there might be even more types or pathways of autophagy.[12]

3.1.2 Autophagy and AD

In the healthy human brain, clearance of misfolded and unused proteins is an important factor to maintain normal cerebral functions. In turn, AD models have shown, that there is a significantly increased accumulation of Aβ-plaques and tau tangles, if autophagy is impaired. In AD, impaired mTOR-dependent and independent pathways contribute to the dysfunction of autophagy.[17] This effect is not limited to this two proteins. In contrast, as autophagous activity declines with ageing and there are more proteins that can misform or create toxic aggregates. Therefore, agents that target a single misfolded protein may be far less effective than drugs that enhance autophagy and clearance of misfolded proteins as a whole.[6]

3.1.3 Importance as a potential therapeutic target

Under normal cellular conditions, the cell enters an autophagous state in an environment of nutrient starvation. Since this is not necessarily an available option, influencing the autophagy-inducing mechanisms is a more viable alternative.

As these underlying cascades are relatively complex, targeting autophagy in a possible treatment strategy for AD requires specific knowledge of the involved pathways and stages. Ideally, a combination of drugs or substances that target more than one involved cascade could be most promising to restore normal autophagous activity in AD patients.

Also, it has shown that simply increasing autophagous activity alone is not necessary beneficial, as it leads to accumulation of autophagosomes and undigested autolysosomes which can block axonal trafficking and lead to axonal swelling[6]. Still, there are some promising substances that support targeting autophagy as a trigger of neurodegenerative diseases:

A widely used autophagy activator is **rapamycin**, (a substance used in transplantational medicine and is safe in humans [LoE: *1a*]) that blocks mTOR-C1-kinase activity, which is elevated in the brain of 3xTg-mice (a triple-transgenic mouse model of AD)[18]. Blocking this pathway also reduced Aβ and tau pathology and significantly reinstated cognitive impairment [LoE: *in vivo*]. Since the mTOR pathway also has functions in several cell processes, such as metabolism and growth, inhibation may involve some harmful side effects. Therefore, more studies are now investigating mTOR-independent pathways to modulate autophagy.[13]

A promising mTOR-independent candidate is **nilotinib** which is currently tested in a phase II trial in AD.[e] Originally an FDA-approved drug to treat adult chronic myeloid leukemia, it boosts autophagous activities by increasing levels of parkin, which is an

[e] ClinicalTrials.gov Identifier: NCT02947893

ubiquitin ligase that plays a critical role in ubiquitination of damaged and misfolded proteins [LoE: *in vivo*].[6,19] Opposed to rapamycin, it induces autophagy via AMPK (5' AMP-activated protein kinase) pathway activation. As this is an mTOR-independent pathway, it is possible that this way of activation shows less harmful side effects.[13]

Further, studies in transgenic mice have shown that **methylthioninium chloride** (methylene blue) is a potent inducer of autophagy. It altered the levels of LC3-II, cathepsin D, BECN1, and p62, which are all autophagy indicators that are involved in regulating the underlying pathways.[20] In doing so, formation of tau tangles could be decelerated [LoE: *in vivo*].

Apart from the therapeutic potential that autophagous processes represent, there also is a need to find more accurate AD-associated biomarkers that are able to reliably quantify autophagic activity in human brains.

3.2 Neuroinflammation

Inflammatory processes, especially of the central nervous system and brain (neuroinflammation), appear to be some of the key processes in neurodegenerative diseases and aging as a whole. On one hand, they are an important tool for the organism to help recover from certain diseases. On the other hand, they often contribute to disease progression itself.

3.2.1 Activation and involved pathways

3.2.1.1 Microglial cells

In the central nervous system (CNS), microglia cells (part of the innate immune system), control inflammatory processes and usually maintain normal CNS function by surveilling their surrounding microenvirenment, maintaining homeostasis and

neuronal integrity. Upon arrival of adverse stimuli, they are activated and respond according to the external stimulation factors.[21]

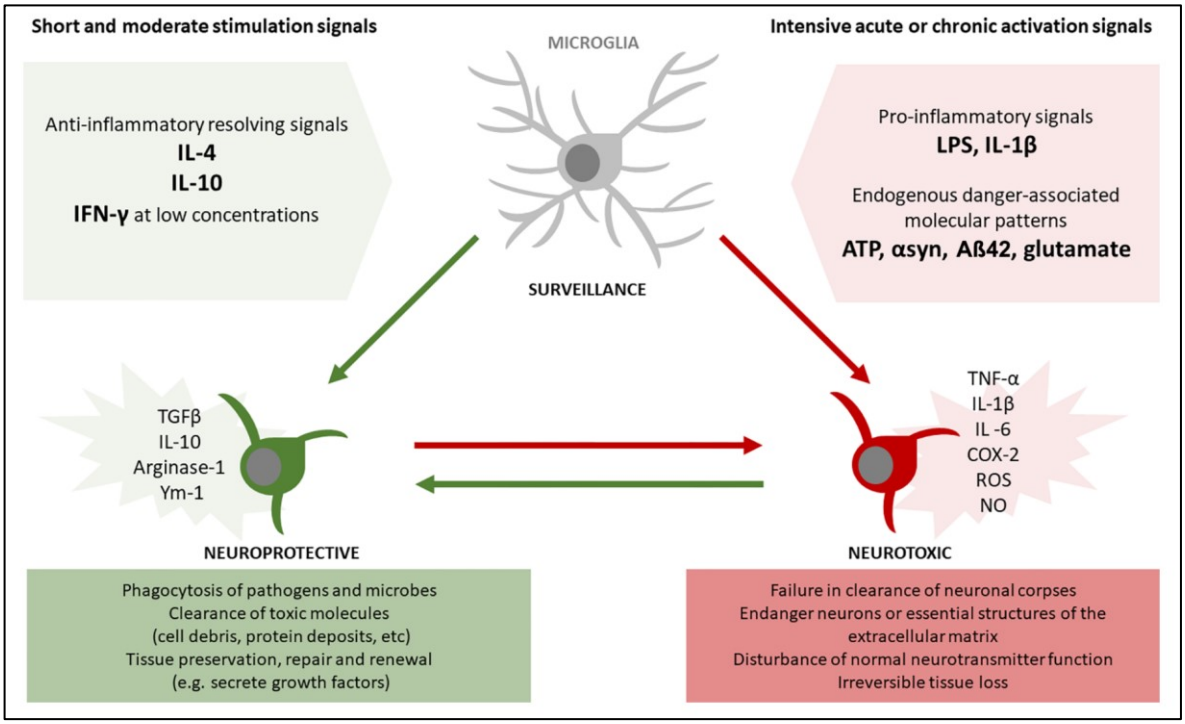

Figure 3: Microglial cells respond differently depending upon type and intensity of activation signals. Edited after Carregosa *et al.* (2019)[21]

Microglial cells behave differently according to the amount and type of activation or damage. Under moderate or transient activation, they act as protective mediators for cells, playing an immune resolving, anti-inflammatory part and supporting their surrounding cells by secreting factors that promote cell renewal (TGF-β, IL-10, Arginase-1, Ym-1 etc.). In this state, they act as neuroprotective mediators. In contrast, when intensive acute or persisting micrglial activation occurs, they secrete different pro-inflammatory cytokines (TNF-α, IL-6, IL-1 β, COX-2) in combination with reactive oxigen and nitrogen species (ROS, NOS). These are substances that promote neuronal damage, disturbe neurotransmitter function and ultimately lead to irreversible tissue loss (Fig. 2).[21]

Toll-like receptors (TLRs) also play a indispensable role in cytokine release and pro-inflammatory processes. They activate and signal their downstream pathway to activate NF-κB and pro-IL-1β, both of which are responsible for neuroinflammation and linked to the pathogenesis of different age-related neurological conditions.[22]

3.2.2 Neuroinflammation and AD

It is understood that inflammatory processes and persistant microglial activation contribute to cellular aging – a connection, for which the term „inflammaging“ has been found.[6] Especially systemic, chronic and low-grade inflammation have shown to be significant risk factors for morbidity and mortality in aging individuals. Systemic inflammatory biomarkers (e.g. IL-6, fibrinogen and C-reactive protein) are associated with a decline in regional cerebral blood flow, cortical thinning and poorer abilities in learning and memory function.[6] Activated microglial cells also lose their phagocytic ability, which further contributes to accumulation of detrimental molecules, that can play a role in AD and other neurodegenerative diseases.[23]

Regarding pathogenetic processes, it is generally accepted that chronic neuroinflammation in AD patients is not caused by senile plaques and tau tangles but has to be understood as an independent process that contributes to AD progression and pathogenesis as much as plaques and tangles.[24] Nevertheless, the two cascades are intertwined as it has shown that Aβ-oligomers can activate several receptors on the microglial cell surface, which then release proinflammatory cytokines. The cross-talk of these cascades combined with pathological accumulation of Aβ is a key factor that drives neuroinflammatory responses in AD.[25,26]

3.2.3 Importance as a potential therapeutic target

Considering the neurotoxic effect of chronic neuronal inflammation, controlling this inflammatory cascade has shown to be a viable target to fight neurodegenerative diseases. Numerous clinical trials utilizing broad-spectrum anti-inflammatory drugs have nevertheless failed to improve outcome with AD patients. This includes non-steroidal anti-inflammatory drugs (NSAID) naproxen, acetylsalicylic acid, celecoxib as well as prednisone, the group of statins, and rosiglitazone [LoE: *1a*].[6,25]

There are some ongoing clinical trials, that target specific factors of the inflammatory cascade:

GC021109, a novel small-molecule compound, promotes microglial phagocytosis by activating the microglial P2Y6 receptor [LoE: *in vitro*]. It has shown to be safe in AD patients in a phase I trial (sponsor: GliaCure, Inc.).[27,28]

Itanapraced (CSP-1103) is a small molecule that induces microglial activation and drives the expression of microglia markers [LoE: *in vivo*]. In primary astrocyte-microglia cultures, it suppressed the expression of TNF-a, IL-1 and NOS induced by Aβ, suggesting its potential therapeutic efficacy as microglial modulator in the early phase of AD.[23] CereSpir, Inc., the sponsoring company, also stated that itanapraced significantly reduced soluble CD40 ligand and TNF-α as well as total tau in a phase II study in humans.[29]

Additionally, it has shown that the autophagy and neuroinflammation cannot be seen as independent processes. Anti-inflammatory effects are in some cases secondary to autophagy induction because autophagy itself possesses anti-inflammatory effects that rely in part on inflammasome inhibition.[30,31]

3.3 Senescence

Cellular senescence describes a state in the cell cycle, in which the cell permanently arrests its cell cycle and stops dividing. It was first discovered in fibroblasts, which stop dividing after about 50 cell pupolation doublings before entering „replicative senescence“. This „hayflick limit“ is a very robust tool of the organism to stop old or damaged cells from accumulating. It paved the way for the exploration of cellular aging and its underlying mechanisms.[32]

Hayflick, which discovered this mechanism in 1961, hypothised that these now nondividing cells were involved in the processes of aging, because they had lost the ability to participate in repairment and regeneration within tissues, which was later

confirmed.[33] After activation of senescence, senescent cells need to be cleared from the organism, so that they don't accumulate. This can be achieved by either selectively destroying those (as done by senolytic drugs) or inhibiting their function (senostatic drugs).[34]

Conclusively, senescent cells exhibit the following properties: irreversible replicative arrest, apoptosis resistance and frequently acquisition of a pro-inflammatory, tissue-destructive senescence-associated secretory phenotype (SASP), where cells produce high levels of inflammatory cytokines, immune modulators, growth factors, and proteases, which in turn facilitates hallmarks of AD such as neuroinflammation.[35] Additionally, they prevent their apoptotic clearance by using, so called pro-survival senescent cell anti-apoptotic pathways (SCAPs) – often targeted in the development of senolytic drugs.[36,37]

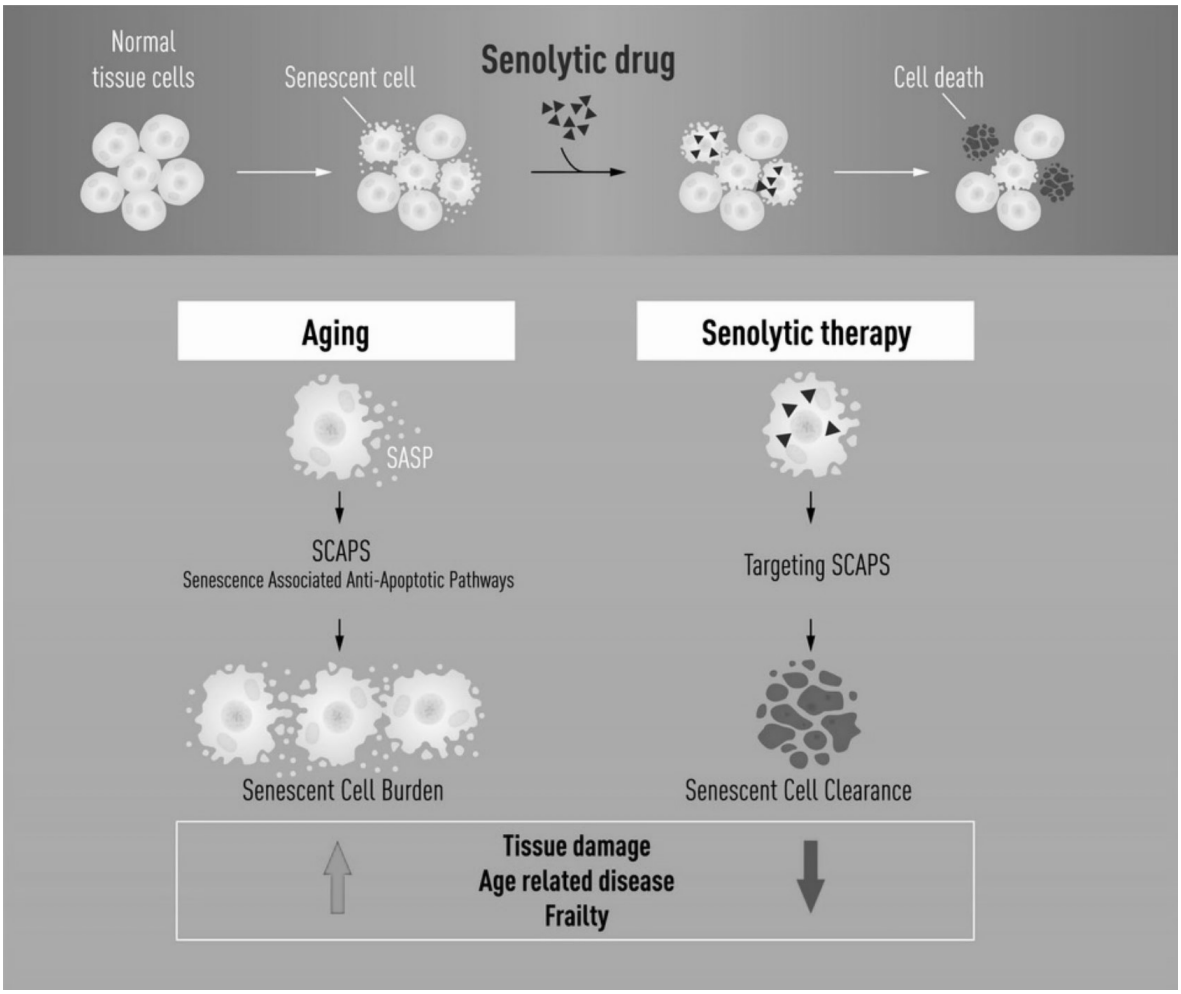

Figure 4: Senescence in aging and senolytic therapy. Edited after Kirkland *et al.* (2020) [37]

3.3.1 Activation and Involved Pathways

Senescence can be induced by a variety of intra- and extracellular factors of cellular stress including abnormal cellular growth, oxidative stress and also autophagous processes. DNA damage, reactive oxygen species (ROS), strong mitogenic signals, depletion of certain tumor suppressors or mitotic stress also induce senescence.[33] In a normal cell cycle and cellular aging, cells stop replicating after about 50 divisions. This is caused by shortening of telomeres (repeating TTAGGG nucleotide sequences at the end of chromosomes), which occurs because DNA polymerases are not able to completely replicate these sequences. As critically short telomeres can lead to chromosomal instability and tumor formation, the cell enters a state of cell cycle arrest and stops dividing.[38]

Disregarding the initiating circumstances, entering the senescent state is coordinated by the p53/p21 and the p16 tumor-suppressive pathways. Baker & Petersen describe the underlying cascade in their 2018 paper as follows:

> *„Uncapped telomeres and DNA double-strand breaks activate a DNA damage response that leads to stabilization of p53 through posttranslational phosphorylation by ATM and ATR serine/threonine protein kinases or by blocking of p53 degradation (…). Transcription of the cyclin-dependent kinase inhibitor (CDKi) p21 occurs upon p53 stabilization, leading to an initial arrest of the cell cycle. After this initial transient arrest, permanent arrest is controlled by $p16^{INK4A}$ transcriptional upregulation through p38 and/or ERK signaling. Once present, $p16^{INK4A}$ inhibits the activity of both CDK4 and CDK6, thereby leading to RB hypophosphorylation and permanent blockage of S phase entry."* [33]

Once the cell enters the state of replicative senescence, this cell cycle state is irreversible, and the cell utilizes SCAPs to stay alive.[8] Also, a certain phenotype of senescent cells has been identified that releases proinflammatory cytokines,[36] which

in turn agein promote processes involved in neuroinflammation (as discussed above).

3.3.2 Senescence and AD

In literature, the link between senescence and AD-inducing pathways is well documented. In patients with neurodegenerative diseases, various markers of senescence have been observed. Also, it has been shown that senescent cells that express the cell cycle inhibitory protein p16 actively drive age-related tissue deterioration and shorten healthy lifespan in mice [LoE: *in vivo*].[39]

In 2018, Bussian *et al.* found a casual link between the accumulation of senescent cells and cognition-associated neuronal loss. In a mouse model of tau-dependent neurodegenerative disease, they were able to show that p16-positive senescent astrocytes and microglial cells accumulate. Upon clearing these cells, several AD-linked biomarkers improved - including gliosis, hyperphosphorylated tau accumulation, and degeneration of cortical and hippocampal neurons [LoE: *in vivo*]. The available evidence therefore suggests that there is a link between the cellular mechanism and several age-related diseases such as AD, atherosclerosis and osteoarthritis.[8]

3.3.3 Importance as a potential therapeutic target

Persisting and accumulating senescent cells not only negatively influence the outlook for neurodegenerative diseases, but have also have been identified as a negative factor for other age-related health factors. In mice, clearance of p16-positive cells delayed tumorigenesis and attenuated age-related impairment of several organs including kidney, heart and fat tissue. In mouse models, knockout of the related genes and subsequent clearance of the senolytic cells increased the healthy life span and occurred without apparent side effects [LoE: *in vivo*].[39]

Whereas gene-knockout is not an option in humans, pharmacological elimination of senescent cells using senolytic drugs could be a promising therapeutic approach. The first senolytic drug (agents that selectively induce apoptosis in senescent cells) was described by Zhu *et al.* in 2015.[40] Since then, more potential candidates have been identified. Currently, they are being tested in proof-of-concept clinical trials. A few notable examples include:

Navitoclax, a small molecule that occupies the inhibitory binding regions of members of the BCL-2 (B-cell lymphoma) family of proteins, which regulate apoptosis. BCL-2 are regarded as protooncogens, promote antiapoptotic processes and hinder clearance of senescent cells. In vitro and in vivo, navitoclax has shown to inhibit their antiapoptotic activity and promote clearance of senolytic cells. Bussian *et al.* showed in 2018 that administration of navitoclax in mice that overexpressed p16-positive cells, resulted in similar effects as knocking out the corresponding gene. Administration of the substance prevented the upregulation of senescence-associated genes and attenuated tau phosphorylation, an important hallmark of AD [LoE: *in vivo*].[8]

Dasatinib + Quercetin: Dasatinib is a small molecule and tyrosine-kinase inhibitor, used in leukemia treatment. Quercetin is a polyphenol compund, more specifically a flavonol, which occurs often in many fruits, vegetables and plants and is known for its antioxidant and tumor-suppressive properties. Both substances are FDA-approved and safe for use in humans [LoE: *1a*]. The substances were selected by Zhu *et al.* through a mechanism-based approach, as opposed to the random high-throughput screening usually used for drug discovery, harnessing their properties that were already known.[40] Interestingly, dasatinib and quercitin interact very cell-type-specific, so that they had to be combined to leverage significant results in different cell types (mouse embryonic fibroblast, human endothelial cells). [36,40,41]
Another very interesting finding in studying dasatinib and quercetin is that they seem to exhibit their senolytic potential also when administered intermittently. Despite their pharmacokinetic elimination half-life of a few hours, a single dose of the substances senolytic activity for at least 7 months [LoE: *in vivo*].[36,40] The frequency of administration therefore will potentially depend on the conditions that induce cellular senescence.

Beside the fact that there may be tremendous clinical potential in senolytic drugs, there are also obstacles that have to be cleared before the full therapeutic potential can be harnessed. As there are practically no direct biomarkers of senescent cells,[42] development of reliable markers is necessary to further harvest the favorable potential of senolytics. Until then, some scientists use a modified version of Koch's postulates of disease, to identify truly senolytic drugs. Kirkland & Tchkonia (2020) formulate it as follows:

> *„If an agent is truly senolytic, then:*
>
> *1) Senescent cells should be present in association with the phenotype*
> *2) Individuals without senescent cells should not have the phenotype*
> *3) Inducing accumulation of senescent cells should cause the phenotype*
> *4) Clearing these induced senescent cells should alleviate the phenotype*
> *5) Clearing naturally occurring senescent cells should alleviate the phenotype*
> *6) The drug should induce few or no effects related to the phenotype in young individuals without senescent cells*
> *7) Administering the senolytic candidate intermittently should be effective and*
> *8) The senolytic candidate should alleviate multiple age-related conditions."*[42]

While senolytic drugs appear to provide beneficial effects in rodent models of aging, they may also be associated with slowed wound healing [LoE: *in vivo*].[33,43] Also, all tested substances exhibit some type of cell-type specifity, so there may not be a single agent to treat all senescent-related pathologies or combination therapies have to be applied [LoE: *in vitro*].[36] Finally, exploration of the underlying mechanisms and reliable and causal linking between senescence and neurodegenerative diseases requires further data collection.

Nevertheless, considering the huge impact of AD on patient's quality of life, the available evidence suggests that keeping senescent cells in scope as a therapeutic target is definetly more than warranted and should be part of a comprehensive treatment approach.

4 Epigallocatechin gallate

4.1 General

Epigallocatechin gallate (EGCG), also known as (−)-epigallocatechin-3-O-gallate, is a polyphenol compund. Chemically, it consists of the ester of epigallocatechin and gallic acid. As a catechin it belongs to the group of flavonoids.

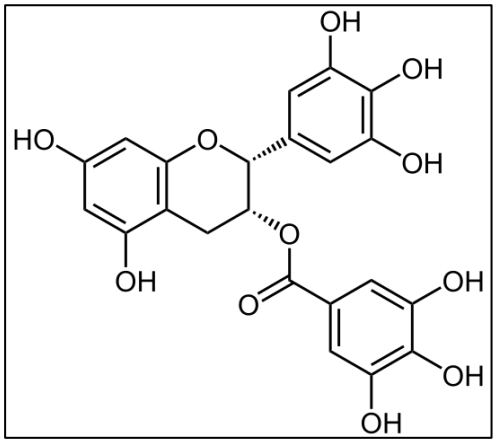

Figure 5: Chemical structure of EGCG [146]

4.1.1 Sourcing

Naturally, it occurs in relatively high concentration in green tea leaves (dried: 7380 mg/100 g), white tea leaves (dried: 4245 mg/100 g), as well as Oolong tea - all obtained from the same plant, *Camellia sinensis* (L.) Kuntze.[44] This plant contains a number of different green tea catechins (GTCs), whereas EGCG is the most abundant and potent one. In black tea, the catechins are mostly fermented to oligomere theaflavins by polyphenol oxidases. EGCG can be relatively easily derived from natural plant sources which are already extensively cultivated.[44]

4.1.2 Usage

Because of it’s antioxidant properties, EGCG is already used as a dietary supplement and it contributes to the popularity of green tea, as it is held responsible for many of it’s beneficial features. EGCG has been studied for its anticarcinogenic, anti-obesity, antidiabetic, and antiinflammatory effects, and also regarding it’s effect on neurodegenerative diseases.[45–47] Additionally, more recent findings suggest even more mechanisms of action for EGCG including interactions with cellular plasma membranes, activation of second messengers and signal transduction pathways, modulation of metabolic enzymes, and autophagy.[48]

4.2 EGCG and AD

Regarding neurodegenerative diseases, several in vivo as well as human observational and intervention studies have shown that there is a inverse link between the amount of green tea catechin intake and cognitive impairment, while others did not.

In a AD mouse-model, Rezai-Zadeh et. al. showed that high doses of EGCG (administered orally or by i.p. injection) significantly lowered Aß pathology and plaques and provided a significant cognitive benefit to the affected mice [LoE: *in vivo*].[49]

In regard to studies in humans, Scholey *et al.* (2012) conducted a double-blind, placebo-controlled crossover interventional study, which found that 300 mg EGCG administration was associated with a significant increase in α-, β-, and θ-brain wave activities [LoE: *2b*].[50] In a double-blind, randomized controlled study conducted in Japan, where participants were given 2 g/day of green tea powder (containing 220 mg of catechins) or for 12 months. However, the results showed that one year of green tea consumption did not significantly affect cognitive function [LoE: *2b*].[51]

Also, a range of observational studies has been conducted in humans in the recent years (see Table 2), with mixed outcomes [all: LoE *2b*].

#	Primary author (year)	Favorable Effect
1	Kuriyama (2006)	Yes [52]
2	Ng (2008)	Yes [53]
3	Huang (2009)	Yes [54]
4	Feng (2010)	Yes [55]
5	Noguchi-Shinohara (2014)	Yes [56]
6	Mashal (2013)	No [57]
7	Nurk (2009)	Yes [58]
8	Wu (2011)	No [59]

9	Feng (2012)	Yes [60]
10	Wang (2014)	No [61]

Table 2: Examples of observational studies on the effect of (green) tea consumption on cognitive impairment. Edited after Pervin *et al.* (2018) [46]

All above mentioned studies focused on cognitive functions as a whole and explored whether EGCG/green tea catechins could be a viable substance to combat or prevent a loss in memory function as a standalone therapy. In the following chapters, the author therefore examines the effect of ECGC on certain contributing pathways of AD, which could prove viable if it is used in a therapy strategy involving multiple substances or as a co-therapy.

4.2.1 EGCG and Autophagy

Apart from the antioxidant properties of green tea catechins, their autophagy-inducing effects have been studied in a variety of settings.

As mentioned in chapter 3.1.3, the mTOR pathway plays a indispensable role in autophagy activation. Holczer *et al.* showed in 2018, that ECGC treatment is able to induce mTOR-dependent autophagy in HEK293T (human embryonic kidney) cells. It further weakens the effect of negative regulators of autophagy (such as GADD34), that control apoptosis. Therefore, EGCG was able to extend autophagy, delaying apoptosis mediated cell death and eventually extending cell viability [LoE: *in vitro*].[62,63]

In endotoxin-stimulated macrophages, optimal concentrations of EGCG were able to induce autophagy and anti-inflammatory effects. EGCG achieved this by inhibiting HMGB1 release and stimulating its autophagic degradation [LoE: *in vitro*].[64]

Grube *et al.* found that ECGC is able to induce autophagous effects on primary human glioblastoma cell cultures. In high but CNS-achievable concentrations (100 µM), the substance (which was applied in pure form as well as a tea extract dietary

supplement) activated autopagy in the brain cancer cells, and triggered different endogenous repair mechanisms to protect the cells. After treatment with very high levels of EGCG (500 µM), strong induction of autophagy and apoptosis was observed [LoE: *in vitro*].[65] This confirms a 2008 study by Hashimoto *et al.*, which suggests that higher concentrations of EGCG do not promote but inhibit autophagy and lead to apoptosis [LoE: *in vitro*]. The macrophage cell lines used in this study were treated with 100 µM and above.[66]

Overall, autophagy-inducing properties of EGCG depend upon the dosage used, level of cellular stress, and the cells lines used. When considering available evidence, the correlation between EGCG and autophagy are not yet fully understood. Nevertheless, conducted studies have shown that EGCG possesses autophagous-inducing properties *in vitro*, and that there is some potential to exert these properties also *in vivo* as well. To shed light on this, more thorough and especially clinical research will be necessary.

4.2.2 EGCG and Neuroinflammation

EGCG is a polyphenol and flavonoid compound - both groups of chemicals that have been found to interfere with cascades that promote neuronal inflammation. Studies have shown that there is a link between flavonoid-rich diets and lower levels of inflammatory biomarkers [LoE: *in vitro*].[67] Poulose *et al.* proved further, that Flavonoids inhibit the production of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1 in microglial cells, suggesting close involvement in pathways such as NF-κB or MAPK [LoE: *in vitro*].[21,68]

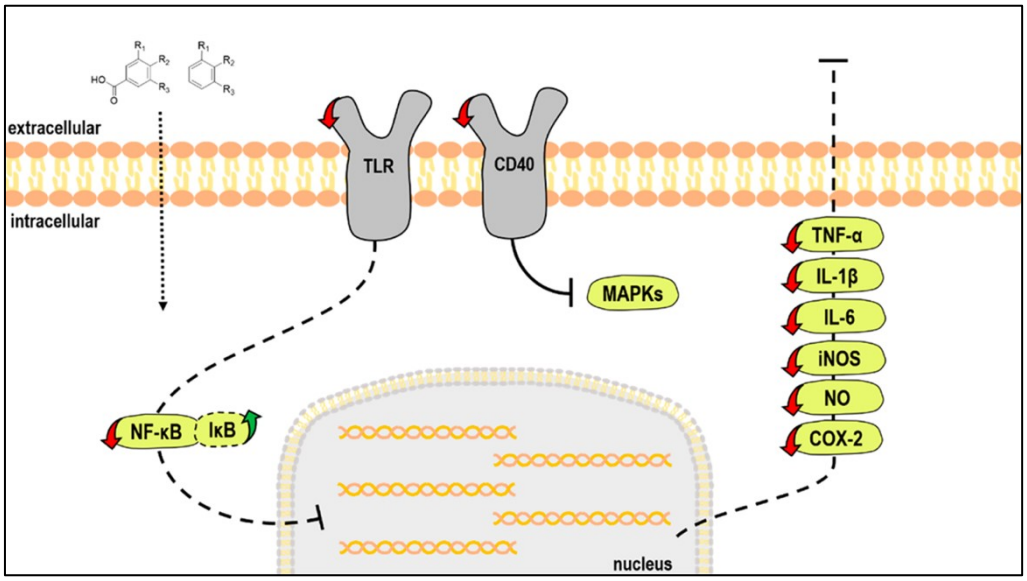

Figure 6: Main molecular targets of low-molecular weight polyphenols metabolites in stimulated microglia cells. Edited after Carregosa *et al.* (2019)[21]

EGCG in general inhibits secretion of TNF-α, IL-6 and IL-8 through the attenuation of ERK and NF-κB in HMC cells[69] [LoE: *in vitro*], which could be beneficial in immune suppression treatment and targeting neuroinflammation.

Apart from this, EGCG seems to be interacting with TLRs, especially with TLR4. Byun *et al.* showed, that EGCG downregulates TLR4 signal transduction (at a low concentration of 1 µM) in macrophages and hindering TLR4 expression through 67LR. This way, it inhibits activation of downstream signaling and consequent inflammatory responses (MAPK and NF-κB activation) [LoE: *in vitro*].[22,70]
These findings were recently fortified in vivo, as Seong *et al.* showed that EGCG supresses TLR4-NF-κB signaling pathway in mice and therefore exhibited neuroprotective effects [LoE: *in vivo*].[71]

Several other *in vivo* studies have come to similar results. Lee *et al.* tested the effects of EGCG on neuroinflammation and amyloidogenesis, in mice with systemic inflammation. The authors demonstrated that EGCG was able to fight CI induced by lipopolysaccharide (LPS) and neuronal cell death. Moreover, EGCG prevented LPS-induced astrocyte-activation and cytokine expression (TNF-α,IL-1β, IL-6) [LoE: *in vivo*].[72,73]

Overall, epigallocatechin gallate shows great potential in fighting neuroinflammation by interfering with its initiating cascades, suggesting that EGCG could be considered a therapeutic agent for neuroinflammation-associated AD. Since polyphenols in general, and EGCG in particular interact with more than one inflammation-triggering compound, there is a great need for controlled trials in humans to explore whether the positive effects shown *in vitro* and animal studies also manifest in humans.

4.2.3 EGCG and Senescence

In protecting the organism from the negative impact of senescence, two properties play an important role: its ability to supress premature senescencee in cells – e.g. through antioxidant or other DNA-protective measures – and the capability of inducing clearance of already senescent cells (called senolytics).

EGCG is widely recognized to possess antioxidant abilities, based upon its chemical structure (i.e. the presence of phenolic groups).[74] Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species (ROS).[75] When ROS are genereted extensively, the imbalance between antioxidant and oxidant substances can damage DNA and induce senescence or cancer.[33]

Numerous studies have shown that EGCG acts as an antioxidant regarding neurodegenerative diseases, and that EGCG is able to scavenge free radical ions and increase the activity of antioxidant enzymes.[75,76] In aging rats, it increases the activity and level of antioxidant enzymes like superoxide dismutase and catalase and non-enzymic antioxidants like tocopherol, ascorbic acid and glutathione. Further, it decreased the levels of protein carbonyl, all of which can prevent age-associated oxidative DNA damage [LoE: *in vivo*].[77]

As previous work has mostly focused on the antioxidant abilities, few researchers have adressed the question of EGCGs direct senolytic ability – one notable exception is a study conducted on H_2O_2-treated senescent preadipocytes. Kumar *et al.* found evidence of ECGCs senolytic ability in concentrations of 50 and 100 µM, in which it downregulated PI3K/Akt/mTOR and AMPK signaling in the cells and also

suppressed ROS, iNOS, Cox-2, NF-κB, SASP and p53 mediated cell cycle inhibition. It further promoted apoptosis of senescent cells by suppressing the accumulation of anti-apoptotic protein Bcl-2 [LoE: *in vitro*].[78]

Conclusively, the senolytic abilities of EGCG remain mostly unclear, especially in regard to neurodegenerative diseases and AD in particular. Further research (especially *in vivo*) is necessary, but some promising research indicates that EGCG is a potential senolytic substance – especially when paired with its mTOR inhibiting abilities.

4.3 Pharmacodynamics & -kinetics

The bioavailability of polyphenols has a high interperson variability, mainly because it is dependent on the degree of polymerisation and epigenetic factors, such as the glycosilation pattern. Natural occuring polyphenols often need to be hydrolized for absorption, where gut microflora plays an important role, as it helps with deglycosilation, dehydroxylation and demethylation.[22] Flavonoids like EGCG further require deglycosylation in the small intestine by β-glucosidases to convert into their aglycone (glucose-free) form and be absorbed.

Because of the high EGCG content of green tea and its widespread consumation, the pharmacological properties have already been extensively studied. The oral bioavailability of EGCG is, despite its flavonoid properties, relatively high and is dose-dependant. This has been shown shown in mice tissues and in a phase I clinical study in humans by Chow *et al*.[79,80] It increased at higher doses, possibly due to saturable presystemic elimination of orally administered green tea polyphenols. A follow-up study found that in doses up to 800 mg/day it is safe to take green tea polyphenol products (an amount equivalent to 8-16 cups of green tea per day).

When taken in higher doses, however, green tea catehins can significantly increase serum transaminases, which indicates hepatotoxicity. Therefore, the European

Food Safety Authority concluded in a 2018 safety assessment that catechins from green tea infusions are generally safe However, EGCG doses above 800 mg/day may pose a health risk and should be avoided.[81] This is especially important when taken as a supplement in pure form, which is already available in doses up to 1000 mg.

Plasma levels reach about 10 µM in animal studies after oral intake of pure EGCG.[80] The ability of a substance to influence the CNS is additionally defined by their own and their metabolites‘ ability to cross the blood-brain barrier (BBB). Studies have shown that polyphenolic compunds are able to cross the BBB in a sufficent manner to unfold their potential activity in the brain. Wu *et al.* demonstrated that the metabolites of EGCG (namely (+)catechin and (−)epicatechin) are able to reach the brain [LoE: *in vivo*].[82] In another study, the amount of BBB-penetration varies between for the tested low-molecular weight polyphenol metabolites [LoE: *in vitro*].[83]

In 2019, Wei *et al.* conducted a study on aging rats that showed signs of cognitive impairment and were given 100 mg doses of ECGC intragastrically. They were able to prove that the brain-blood barrier changes it’s permeability upon aging and onset of cognitive impairment. This blood-brain barrier dysfunction (also called „leaky brain syndrome“) could both increase the risk for neurodegenerative diseases,[84] but in the present study, it also enables significantly more EGCG to reach the CNS, where it can unfold its potential positive properties [LoE: *in vivo*].[85]

Bioavailability of EGCG can be further improved by applying it in a pro-drug form (fully acetylated EGCG, called pEGCG). The study was conducted on neuroblastoma SH-SY5Y cells, and it demonstrated an improved protection by pEGCG over EGCG, most likely due to the activation of the Akt pathway and reduced caspase-3 activity.[22,86]

Generally has to be noted that the bioavailability of flavonoids is hard to mimic in a tissue model *in vitro*. As Kroon *et al.* point out, the findings can often not be directly translated into animal or human applications, as there is a) great variation in the concentration of compunds at the site of action and b) polyphenols often undergo extensive chemical modification before they reach their destination. Therefore, there

is a great need to conduct more trials in animals and humans, to strengthen the theories and results gained from *in vitro* experiments.[87]

4.4 EGCG: Clinical potential

Conclusively, EGCG alone may not be a viable option to treat AD as a standalone-therapy. The evidence does not support EGCG as a monotherapy in AD or memory loss and the full picture remains unclear, since there is a lack of suitable and reliable RCTs in humans. To prove or disprove a direct link to AD, study designs needs to be adapted, since conducted research mostly evolved around tea consumption as a whole and did take into account confounding or environmental factors, or direct EGCG usage as a pure substance. Therefore, more rigorous human studies are required to understand the overall neuroprotective effect of green tea catechins and EGCG in particular.

Nevertheless, the existing results certainly warrant the further exploration of using EGCG in a therapy strategy involving multiple compounds and targeting more than one hallmark of the disease. Especially recent findings regarding its capability to inhibit neuroinflammation are promising and are solid enough to warrant further application of EGCG against AD and neurodegenerative diseases.

Regarding the autophagy-inducing and senolytic capabilities, further research in cell cultures and animal models needs to be conducted, which then needs to be proven in solid, well designed RCTs.

Another challenge in using EGCG is the question of bioavailability of the compound at the site of action (= in the CNS). Nonetheless, promising studies have been conducted in this regard (such as applying EGCG as a pro-drug), which give a positive outlook on this issue.

5 Fisetin

5.1 General

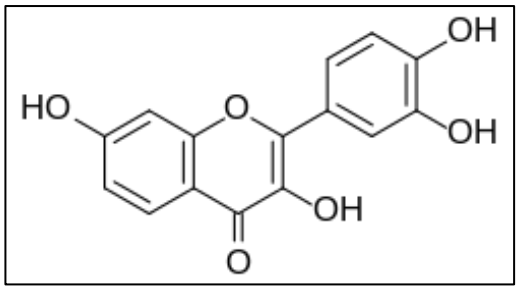

Figure 7: Chemical structure of Fisetin [147]

Fisetin (chemically: 7,3′,4′-flavon-3-ol) is a flavonol compund, and a member of the flavonoid group of polyphenols (as EGCG).

5.1.1 Sourcing

Fisetin can be easily gained from plants and is contained in a variety of foods like fruits and vegetables, where it serves as a coloring agent (yellow-toned). The highest concentrations in plant sources are found in strawberries (160 µg/g), apples (26 µg/g) and persimmon (10 µg/g).[88] It is further contained in wine, tea infusions of the mentioned substances and onions.[89]

5.1.2 Usage

In 1891, fisetin was first described. Since then, it has mainly been used as a coulouring agent. Research of its potential positive health effects have only gained traction in the last 20 years. In 2001, Ishige *et al.* identified fisetin in a screen for flavonoids that are able to prevent oxidative stress-induced nerve cell death and subsequently its antioxidant capabilities came into focus.[90] Now, it is available as a dietary supplement in pure form, in doses up to 500 mg, marketed to enhance brain health. In recent years, it has achieved the image of a *functional food* and is promoted to prolong lifespan and counter various effects of aging. Apart from its antioxidant abilities, fisetin has been studied for its wide ranging effects on a number of key pathways involved in cell cycle regulation, apoptosis, the suppression of inflammation, angiogenesis, and metastasis.[34]

5.2 Fisetin and AD

Since fisetin has been only studied extensively in the last 20 years, there are little to no observational or interventional studies in humans available that describe a direct link between AD and fisetin.

In vivo, fisetin has been found to enhance long-term memory in non-AD mice (orally administered, 10-25 mg/kg) [LoE: *in vivo*].[91] In an $A\beta_{1–42}$ mouse-model of AD, Ahmad *et al.* were able to show that fisetin significantly decreased the $A\beta_{1–42}$-induced accumulation of Aβ, BACE-1 expression, and hyperphosphorylation of tau protein. It reversed synaptic dysfunction and had a favorable effect on different proteins involved in AD pathology. Further, it also suppressed various neuroinflammatory mediators (see section 5.2.2). Ultimately, it improved mouse memory when administered intraperitoneally (20 mg/kg/day for 2 weeks) [LoE: *in vivo*].[92]

5.2.1 Fisetin and Autophagy

Research on fisetin and autophagy mainly focused on cell cultures and other *in vitro* techniques. The conducted studies adressed mainly the anti-proliferative activity of different flavonoids against various cancer cell types but did not test for favorable effects on neurodegenerative diseases.

Suh *et al.* tested the effect of fisetin on different types of human prostate carcinoma cell lines. Treatment of cells with fisetin inhibited mTOR activity and downregulated several carcinogenesis-involved proteins that resulted in loss of mTOR complex-formation. Fisetin also activated the mTOR repressor TSC2 through inhibition of Akt and activation of AMPK. However, they showed that fisetin treatment leads to induction of autophagic-programmed cell death rather than cytoprotective autophagy as shown by small interfering RNA Beclin1-knockdown and autophagy inhibitor. As the study author noted, this could be worthy in carcinoma treatment, but may be detrimental in treating neurogenerative diseases like AD [LoE: *in vitro*].[93]

Another *in vitro* experiment, conducted by Park *et al.* (2019), tested whether fisetin can induce autophagy in oral squamous cell carcinoma. Various concentrations (10-300 µM) of fisetin were applied to the cells, which then induced autophagous processes significantly, but simultaneously increased apoptotic cell processes. When applying fisetin in combination with an autophagy inhibitor (in this case, the substance SP600125), apoptosis was elevated further [LoE: *in vitro*].[94] In case of oral squamous cell carcinoma, this combination seems very promising – in case of neurodegenerative diseases however, it is not reasonable to raise apoptosis in this manner.

Kim *et al.* showed in 2016, that fisetin stimulates autophagic degradation of phosphorylated tau via the activation of transcription factor EB and Nrf2 transcription factors and that fisetin reduces sarkosyl-insoluble tau levels in mouse cortical cells. Treatment of cortical cells or primary neurons with fisetin resulted in significant decreases in the levels of phosphorylated tau. The researchers found out, that the activation of autophagy was carried out using the mTOR pathway. Further, the level of LC3-II (a autophagy marker) and autophagy-related gene (ATG) products in cells treated with 5 µM and 10 µM of fisetin were significantly increased [LoE: *in vitro*].[95]

In vivo, the effect of fisetin on Pb-induced neurotoxicity in mice was analysed in a 2019 paper (Yang *et al.*). Fisetin supplementation increased protein expressions and promoted the Pb-induced autophagy in brains of mice ($P < 0.05$). However, no significant difference in these autophagy-related proteins was found between the control group and the group of 50 mg/kg fisetin treatment [LoE: *in vitro*].[96]

5.2.2 Fisetin and Neuroinflammation

Regarding the ability of fisetin to inhibit neuroinflammation, numerous *in vitro* and *in vivo* studies have been published (table 3).

LoE	Model / Study population	Mode of action / Result	Ref.
In vitro	HMC-1 mast cells	Inhibits cell-cell communication, NF-κB and MAPK	97
In vitro	Lipopolisaccharide-stimulated mouse macrophages	Suppressed activation of NF-κB and JNK MAPKs, but not ERK	98
In vitro	IH-3T3 and KF8 cells	Enhanced and sustained activation of ERK and JNK but not p38 in response to TNFα	99
In vitro	BV-2 microglial cells	Reduction of microglial activation, PGE2 & NOS production; downregulation of genes for COX2 and IL-1β	100
In vivo	APPswe/PS1dE9 double transgenic AD mice	Prevented memory deficits, increased ERK phosph., decreased oxidative stress, downregulation of p25	101
In vivo	$A\beta_{1-42}$ AD mouse model	Downregulated expression of inflammatory mediators p-IKKβ, NF-κB, TNFα,and IL-1β; hindered Aβ accumulation & tau hyperphosphorylation	92
RCT,1b	colorectal cancer patients	Reduction of IL-8	102

Table 3: Anti-inflammatory effects of fisetin in various studies

In vitro, fisetin exhibits significant effects on multiple inflammatory markers. Its ability to interfere with the release of cytokines and other pro-inflammatory substances has been tested in various cell culture models. Notable examples include HMC-1 mast cells which were stimulated by activated T-cell membranes, where fisetin suppressed cell spreading and gene expression. The stimulation also induced activation of NF-κB and MAPKs. These activations were suppressed by fisetin.[97] As shown by Zheng *et al.*, it reduced microglial activation, and inhibited gene expression of TNF-α, IL-1β, COX-2 and inducible iNOS at both mRNA and protein levels.[100]

Subsequently, researchers were able to confirm the findings in animal models. In an AD-mouse model, intraperitoneal injections of fisetin at a dose of 20 mg/kg/day for two weeks showed a favorable effect not only on $A\beta_{1-42}$ induced memory deficits, but also suppressed various activated NI markers (p-IKKβ, NF-κB, TNFα,and IL-1β), which suggests a clear neuroprotective effect.[92] In another study, Currais *et al.* showed that oral administration of fisetin in APPswe/PS1dE9 double transgenic AD mice from 3 to12 months of age not only prevented memory deficits, but also

increased ERK phosphorylation and reduced p25. Elevated levels of p25 cause disregulation of cyclin-dependent kinase 5 (Cdk5) activity, that leads to neuroinflammation and neurodegeneration.[101]

In humans, the efficacy of fisetin supplementation vs. placebo on the inflammatory status in patients with colorectal cancer has been assessed. 18 patients received 100mg fisetin for seven consecutive weeks. Significant changes were observed in IL-8 concentrations in the fisetin group when compared with the placebo group ($p < 0.03$), which suggests that fisetin could improve the inflammatory status in humans [LoE: *1b*].[102]

Additionally, there are currently two ongoing clinical trials, examining the impact of fisetin on inflammatory processes. In the AFFIRM-LITE study (phase II), researchers test the efficacy of fisetin vs. placebo in reducing inflammatory factors and related measures in 40 elderly adults.[a] In another phase II trial, the effect of fisetin on mesenchymal stem cell function, kidney function, markers of inflammation, and physical function in patients with advanced chronic kidney disease are examined (30 participants).[b] In both trials, fisetin is administered orally, in doses of 20 mg/kg/day for 2 consecutive days (= approx. 1400mg/d for a 70 kg person, which is 10 to 15-fold higher as in the trial mentioned before ([102])). Hope of the researchers is, that the safety of fisetin in higher doses will be demonstrated to permit further clinical trials. No results have been published yet.

In a very recent paper by Ates *et al.* (2020), promising results were obtained using a fisetin derivative. The substance, CMS121, is a small molecule derived from fisetin, which is developed by a drug discovery paradigm based on phenotypic screening assays. In a transgenic mice model that mimics neurodegenerative diseases, CMS121 alleviates cognitive loss, modulates lipid metabolism favourably and reduces inflammation and lipid peroxidation in the brains of transgenic AD mice.[103]

[a] ClinicalTrials.gov Identifier: NCT03675724

[b] ClinicalTrials.gov Identifier: NCT03325322

Conclusively, study results regarding the anti-inflammatory properties of fisetin look very promising, and warrant further studies. Not many researchers have adressed the question if fisetin also exerts protective qualities on the CNS itself, but *in vivo* studies show a clear correllation and positive effects on long-term memory. Also in the few human trials that have been conducted, fisetin delivers promising results in regard to neuroinflammation by suppressing inflammatory markers.

5.2.3 Fisetin and Senescence

Fisetin as a plant-derived flavonoid is considered a natural occuring senolytic and has shown its ability to disrupt senescence in multiple studies and experiments. This is especially interesting as in-deep research of the topic has only gained importance in recent years.

Senolytic flavonoids as quercitin and fisetin act in part by inhibiting BCL-2 family members such as BCL-xL as well as HIF-1a and other senescent cell anti- apoptotic pathway (SCAP) network components [LoE: *in vitro*].[42] In comparison to other flavonoids, fisetin is twice as potent as quercetin in its senolytic properties.[34]

In vitro, Zhu *et al.* showed that fisetin selectively induces apoptosis in senescent (but not proliferating) human umbilical vein endothelial cells (HUVECs). It is not senolytic in senescent IMR90 cells, a human lung fibroblast strain, or primary human preadipocytes, whcich indicates a strong cell type specifity.[104] Interestingly, the fisetin plasma concentrations achieved in a mouse study (2.7- 349.4 µM)[105] were similar or higher than those Zhu *et al.* found to be senolytic in cultured HUVECs. Thus, fisetin has sufficient bioavailability to reach the site of action in the body [LoE: *in vitro*].[104]

Yousefzadeh *et al.* conducted research on a panel of flavonoid polyphenols, which were screened for senolytic activity in senescent fibroblasts. Fisetin was identified as the most potent because it reduced senescence in murine and human adipose tissue and demonstrated cell-type specificity [LoE: *in vitro*]. It was consequently tested in aged wild-type mice to determine its effect on senescence markers, age-

related histopathology and other disease markers. When administered late in life, it restored tissue homeostasis, reduced age-related pathology, and extended median and maximum lifespan (fisetin dosage: 100 mg/kg orally) [LoE: *in vivo*].[106]

Regarding human trials, currently there are two clinical trials running, harnessing benefit from the senolytic abilities of fisetin. One RCT is conducted to determine if senolytic drugs (dasatinib+quercetin vs. fisetin vs. placebo) reduce senescent cell burden and reduce bone resorption markers/increase bone formation markers to improve skeletal health in elderly women.[a] Another phase II RCT is conducted to evaluate the clinical efficacy of fisetin in symptomatic knee osteoarthritis patients. The main objectives are to determine the safety of fisetin during dosing and whether it reduces senescent cells, pro-inflammatory and SASP markers, and reduces OA-symptoms.[b] In both trials, fisetin is administered orally, in doses of 20 mg/kg/day, for two ([b]) or three ([a]) days. No results have been published yet.

With recent knowledge and ongoing trials in humans, fisetin is increasingly becoming accepted as a potent senolytic agent, that exhibits its potential on certain cell types, as well as in animals and humans. As many studies have focused on the senolytic abilities of fisetin in general, there are is still a knowledge gap of fisetin in relation to AD and neurodegenerative diseases that will hopefully be closed in the following years.

5.3 Pharmacodynamics & -kinetics

Fisetin exhibits dose-dependent bioavailability. Shia *et al.* have investigated the pharmacodynamic properties *in vivo.* After intravenous administration of fisetin (10 mg/kg), the mean plasma concentration–time profiles of fisetin and its metabolites showed a rapid decline of fisetin. The biotransformed metabolites (sulfates, glucuronides) showed higher concentrations than the parent compound at all times, which hints at an extensive biotransformation in the liver. When administered orally,

[a] ClinicalTrials.gov Identifier: NCT04313634

[b] ClinicalTrials.gov Identifier: NCT04210986

(50 mg/kg), fisetin levels remaind stable after the first pass of intestine and liver.[107,108] Maximum plasma concentrations reached 2.5 µg/ml at 15min, with a phase 1-half-life of 0,09h and a terminal half-life of 3,1h after intraperitoneal administration in mice [LoE: *in vivo*].[105] Following oral administration at 50 mg/kg, the serum concentration of fisetin sulfates/glucuronides was maintained at approximately 10 µM for 24 h [LoE: *in vivo*].[109]

It was shown that even after biotransformation, fisetin retains its antioxidant capacity (tested upon inhibiton of (2,2'-azobis(2-amidinopropane hydrochloride) AAPH-induced oxidative hemolysis) signifying that residual phenolic groups are preserved [LoE: *in vivo*].[107] Mohapatra *et al.* conducted a study on the behaviour of fisetin in lipid bilayer liposome membranes and were able to show that fisetin penetrates membranes, but stays near the (polar) head reagion of the lipid bilayers, which may have implications in the antioxidant activity of fisetin [LoE: *in vitro*].[110]

Ragelle *et al.* created a nanoemulsion that raised the bioavailability of fisetin 24-fold when administered intraperitoneally, compared to free fisetin (which proved helpful in treating Lewis lung carcinoma-bearing mice as lower doses were needed).[111]

Generally, few researchers have dealt with the question of brain-barrier permeability of fisetin, and its (or its metabolite's) ability to reach the CNS, and there is still an ongoing debate. In a 2009 study on MDR1-MDCK cell monolayers, it was shown that fisetin exhibited high brain uptake potential [LoE: *in vitro*].[109] Taking into account these findings and results from other *in vivo* studies, in which fisetin was able to enhance memory function in rats and mice, it can be assumed that an effectual amount of fisetin (or its metabolites) is able to reach the CNS.[91,92] In unpublished results, Maher found that sulfated and/or glucuronidated forms of fisetin reach concentrations of 30 µM in the cerebrospinal fluid with a plasma half life of 8 hr in macaques fed a single oral dose of 25 mg/kg.[112]

Regarding the phenomenon of „leaky brain barrier", fisetin seems to exhibit some favorable neuroprotective effects. In 2018, Zhang *et al.* were able to show in an animal study, that fisetin generally prevented extravasation of brain tissue (after traumatic brain injury). Fisetin significantly decreased brain water content and

reduced pathological brain barrier permeability [LoE: *in vivo*].[113] Whether this protective effect can also be drawn over to exhibit neuroprotective effects in AD and neurodegenerative diseases, further research is necessary.

Adressing safety and toxicity, fisetin showed no indications of toxicity in mice, even in doses up to 2000 mg/kg or in long-term feeding (~25 mg/kg for 9 months).[112] In humans, there were no safety issues at doses of 100mg/day.[102]

5.4 Fisetin: Clinical potential

Recent discoveries show that fisetin has positive effects on a variety of pathways associated with neurodegenerative diseases. While the ultimate effects of fisetin on the CNS in humans are still under investigation, it possesses promising characteristics to become part of a therapy approach involving multiple substances.

Results show that may be superior to other senolytics currently in clinical trials. Clinical and pre-clinical data are very encouraging and suggest that most of its properties should be translatable from animal studies into humans. Further, evidence that fisetin is a true senolytic in terms of Koch‘s postulates is mounting.

As a natural compound, occuring in a variety of fruits and vegetables, it is already available as a dietary supplement and has an assuring toxicity profile in humans. All of these observations suggest that fisetin might be of great use in the clinic. Still, to further strenthen the data base, well-designed trials along with the development of reliable analytical biomarkers are required.

6 Spermidine

6.1 General

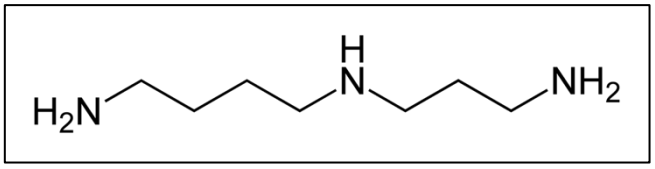

Figure 8: Chemical structure of spermidine [148]

Spermidine, also called monoamine-propylputrescine, is a biogenic polyamine and intermediate product in the synthesis of spermine (which is also biologicalls active). It occurs in all living organisms and plays a role in cellular growth. Polyamines are essential for cell growth and tissue regeneration. They bind and stabilize DNA, modulate enzymefunctions and are required to regulate translation.[30] Because the concentration of spermidine decreases in organisms of age it has been connected to cellular aging.[114]

6.1.1 Sourcing

In living organisms, spermidine is found in ribosomes, where it fulfills multiple metabolic functions. Originally, it was isolated from human sperm in 1878 (av. content: 31 mg/l)[115], which also gave the substance its name.[116] It can also be easily sourced from food, especially from foods composing the mediterranean diet. Dietary sources of spermidine are whole grains, cheese, mushrooms or legumes. Wheat germ has the highest spermidine content of foods (243 mg/kg)[117] followed by soybeans (207 mg/kg)[118].

6.1.2 Usage

Spermidine is currently studied as a potential cure for a variety of diseases. As a caloric restriction mimetic it triggers effects similar to fasting regimens, such as intermittent fasting or caloric restriction, which are well-documented to be beneficial for health and life span extension.[30] Currently, it is available as a food supplement

in pure form, where it is advertised as life-prolonging agent. However, there is no (EMEA or FDA) approved drug available until now.

The life span extending properties of spermidine have been studied *in vivo*, where spermidine was able to prolong the lifespan of *Drosophila melanogaster* fruit flies and that of mice of up to 25%, when administered lifelong (Fig. 9).[119] With the *Bruneck Study*, Kiechl et al. have conducted a prospective population-based study over 20 years (1995-2015) to test the potential association between spermidine content in diet and mortality in humans. The community-based cohort study included 829 participants and was able to show, that deaths per 1000 person-years decreased across thirds of increasing spermidine intake from 40,5 to 23,7 and 15,1. The difference in mortality risk between the top and bottom third of spermidine intakes was similar to that associated with a 5,7 years younger age [LoE: *2b*].[120]

Further, spermidine is studied for its effects on tumorigenesis, cardiovascular and muscle-related diseases and metabolic syndromes.

6.2 Spermidine and AD

The link between Spermidine and AD / cognitive decline has been well established. In numerous animal studies it has shown that spermidine dose-dependently improves performance in most learning tasks, up to a given level from which on performance declines again [LoE: *in vivo*].[121] In *Drosophila melanogaster* fruit flies, spermidine supplementation not only showed significant improvements in short and medium-term memory compared to the control group, but also decreased signs of senile dementia and restored memory performance [LoE: *in vivo*].[114,122]

These findings have subsequently been translated into clinical trials.
In the preSmartAge trial (phase IIa), 30 cognitively intact participants with subjective cognitive decline orally received 1,2 mg of spermidine (daily for 3 months) to evaluate the impact of spermidine supplementation on memory performance in adults at risk for the development of Alzheimer's disease. It was shown that memory

performance was enhanced in the spermidine group compared to placebo [LoE: *1b*].[123,124] The results led to a follow-up RCT (SmartAge, phase IIb), where 100 participants are tested in a monocentric, randomized, double-blind, placebo-controlled setting (dosage: 0,9 mg spermidine + 0,5 mg spermine per day, for 12 months). The effects on cognition and relevant biomarkers in older adults with subjective cognitive decline are measured.[125] The trial is still ongoing, and no results have been published yet.[a]

In 2019, Pekar *et al.* were able to prove the relation of spermidine to age and memory performance. In Austria, serum spermidine levels were correlated to performance in mental state examinations in 80 elderly adults. In this multicentric placebo-controlled study, the participants received spermidine-enriched food, and results demonstrated a positive correlation between enhanced memory performance and serum spermidine level [LoE: *1c*]. The authors therefore suggest the inclusion of spermidine levels as a biomarker in the diagnosis of AD.[126]

The inclusion as a biomarker is further warranted by the findings of Joaquim *et al.*. The researchers were able to show that in AD patients with mild cognitive impairment, the serum spermidine levels are declined and that dynamic changes in the CNS polyamine levels contribute to the cognitive deficit in AD patients.[127]

Conclusively, there seems to be a link between the formation of neurodegenerative diseases and corresponding spermidine levels in the human body. Whether this relationship is causal or a byproduct of other detrimental processes remains unclear and needs to be studied further.

6.2.1 Spermidine and Autophagy

The capability of spermindine to induce autophagous processes – especially macroautophagy – is suggested from an array of studies conducted in recent years.

[a] ClinicalTrials.gov Identifier: NCT03094546

in 2009, Eisenberg *et al.* showed in a comprehensive study that spermidine induced autophagy in multiple model organisms, such as yeasts, flies, nematodes and mammals. The researchers tested, if the *a priori* known life span extending properties were due to the induction of autophagy, and confirmed this assumption using genetically modified, autophagy-deficient Δatg7 fruit flies, in which spermidine exhibited no life-prolonging features (dosage: 0,01 – 1 mM; see Figure 9).

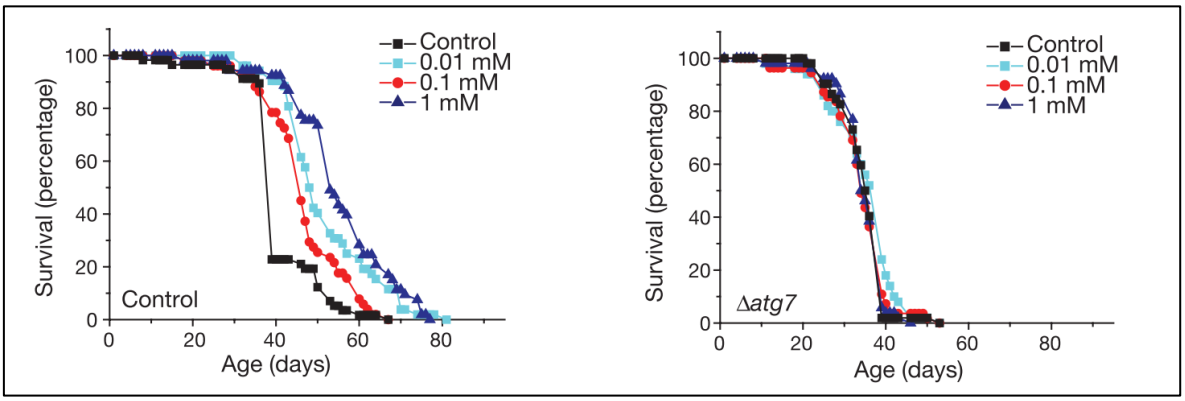

Figure 9: Various concentrations of spermidine prolong the life of *D. melanogaster* (left), but not if the flies are genetically not capable of autophagy (Δatg7 Mutant, right). Edited after Eisenberg *et al.* (2009) [128]

Additionally, they concluded that several autophagy-related genes (most significantly ATG7) were upregulated after spermidine treatment, and that spermidine induces the formation of a signalling protein into newly formed autophagosomes [LoE: *in vivo*].[128] In a follow-up study in mice, Eisenberg *et al.* displayed that spermidine also extends life-span in mice, and additionally exhibits autophagy-dependent cardioprotective features [LoE: *in vivo*]. In humans, the researchers correlated spermidine-rich diets with lower blood pressure, but it is not known whether this correlation is causal or not.[129]

Morselli *et al.* found out that spermidine and resveratrol (a polyphenol mostly found in red grapes) can stimulate autophagy synergistically, both in cultured human cells, and in mice. This was achieved by spermidine without targeting the mTOR-pathway, and thus may have less-harmful side effects than autophagy-activation by EGCG or fisetin. In mice, the effect was measurable using intraperitoneal injected spermidine doses of 50 mg/kg, but there was no pro-autophagic effect in doses of 5 mg/kg [LoE: *in vivo*].[130]

So far, previous work has been limited to *in vitro* and *in vivo* studies. It has not been explored if the beneficial effects found in observational studies and RCTs in humans can be attributed to its pro-autophagic capabilities – which are nevertheless very promising in animals. It is of particular interest to correlate plasma spermidine levels and dietary patterns with the amount of autophagic flux and protein acetylation, which can already be determined. This way, a correlation between spermidine, autophagy and overall health would be possible.

6.2.2 Spermidine and Neuroinflammation

The research of the anti-inflammatory prospects of spermidine, especially regarding neuroinflammation, have not gained as much attention as its pro-autophagic capabilities. However, in recent years this area has also gained in research interest. There are promising findings available, which show that spermidine (and spermine) supress the expression of several pro-inflammatory cytokines in varios research settings (see Table 4).

LoE	Model / Study population	Mode of action / Result	Ref.
In vitro	Murine BV2 microglia cell cultures	Production of inflammatory markers and cytokines decreased upon spermidine treatment	[131]
In vitro	Lipopolisaccharide-stimulated mouse macrophages	Reduced level of pro-inflammatory mediators and cytokines	[132]
In vitro	THP-1 Monocytes	Increased expression and activation of PTPN2, which supresses IFN-γ activated inflammatory response	[133]
In vitro	Human THP-1 monocytes	Anti-inflammatory effects and increased expression and activity of PTPN2	[133]
In vivo	Mice with ear edema	Decreased ear thickness, mediators of inflammation markers and neutrophil infiltrations	[132]
In vivo	Zebrafish *(Danio rerio)*	Prevention of LPS-induced NO production, decreased ROS accumulation and reduced inflammatory cells recruitment	[134]

Table 4: Anti-inflammatory effects of spermidine in various studies

Of particular interest are results, that can be connected to low-grade, age-associated inflammatory processes, that are connected to the emergence of neurodegenerative diseases. According to Eisenberg *et al.* and Zwighaft *et al.*, spermidine and other polyamines play a role in this process as they suppress inflammatory cytokines as TNF-α and others, that induce chronic low-grade inflammation. This could be shown when spermidine was orally administered in mice [LoE: *in vivo*].[129,135]

In humans, blood spermine levels (the main metabolite of spermidine) inversely correlated with the amount of LFA-1 (a protein that mediates adhesion and migration of leukocytes in immune and inflammatory processes), which further confirms the involvement of polyamines in inflammatory processes [LoE: *2b*].[136] The originating mechanisms could lye in epigenetics, for example age-associated changes in DNA methylation patterns. Another possible explaination could be the findings of Yang et al., that indicate that polyamines possess inhibitory effects on pro-inflammatory transcription patterns, such as NF-κB in macrophages [LoE: *in vitro*].[30,137]

Additionally, spermidine was found to suppress overproduction of ROS, and the amount of necrotic cell death, which could be inhibited in yeast and human immune cells by adding exogenous spermidine [LoE: *in vitro*].[128] When necrotic cell death occurs, intracellular compounds including ROS ans cytokines leak out of the cell, resulting in local inflammation.

Overall, results point in a favorable direction, even if there is still some more research necessary to prove a direct or indirect inhibitory effect of spermidine on inflammatory processes. There is especially a need for human trials to further translate the *in vitro* and *in vivo* findings into clinical application. Additionally, it is important to gather evidence regarding the role of spermidine in *neuro*inflammation which may be influenced differently by spermidine than inflammatory processes as a whole.

6.2.3 Spermidine and Senescence

Currently the impact of spermidine on the cellular process of senescence is not well understood. Present research places spermidine, or polyamines as a whole, at the crossroads of autophagy and immune senescence, which contributes to their life-prolonging properties. Despite that spermidine itself does not seem to exhibit senolytic or senostatic capabilities, it seems to have indirect effects on the involved processes, as the following papers show.

For example, Kibe *et al.* (2014) hypothesized that the previously found enhanced longevity in mice may possibly be traced back to the repression of senescence by increased polyamine concentration in the intestinal tract.[138] Matsumoto *et al.* also contemplate that spermidine and other polyamines are able to inhibit chronic low-grade-inflammation, and therefore possibly hinder the activation of cellular senescence [LoE: *in vitro*].[139]

García-Prat *et al.* further explain the indirect senolytic capabilities of spermidine as they describe them as dependent on autophagy in stem cells (Fig. 10). Spermidine administration reversed the age-associated decline of autophagy in muscle stem cells (satellite cells) in mice, preventing them from entering a senescent state [LoE: *in vitro*].[30,140]

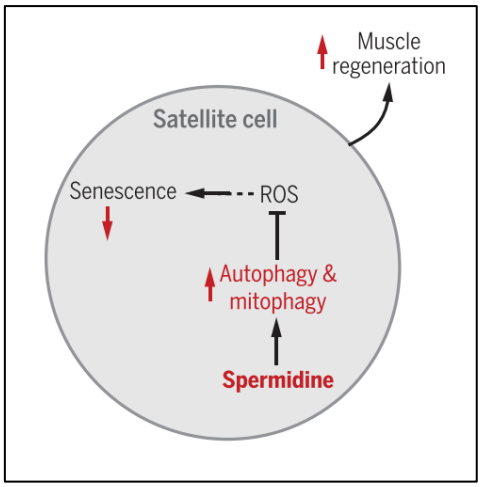

Figure 10: Spermidine-induced suppression of stem cell senescence depends on autophagy (in satellite cells). Edited after Madeo *et al.* (2018) [30]

Taking together the available evidence, spermidine does not qualify as a senolytic for itself, partly also because of the lack of reliable biomarkers that could show a connection to the involved processes. Current research only connects it to other cellular processes such as autophagy or the inhibition of inflammation, that themselves influence and prevent senescence.

6.3 Pharmacodynamics & -kinetics

Spermidine, is a polyamine (as spermine and putrescine) that occurres naturally in the human body. As an endogenous substance, it is involved in several essential cellular functions, as cell growth regulation and proliferation and is therefore present in almost all tissues and organs. Tissue polyamine concentration is determined by endogenous biosynthesis, activity of gut microbota, and food intake and declines with age.[30,124] In the CNS, high concentrations of polyamines are present, which accumulate in synaptic vesicles and are released upon depolarisation.[17]

In the preSmartAge study (also see section 6.2), a randomized, placebo-controlled, double-blind phase II trial, safety and tolerabilty of spermidine was examined in Germany. 30 participants of older age, that were at risk to develop AD, received spermidine-rich plant extract for 3 months, at doses equal to 1,2 mg spermidine per day. The study participants did have better memory performance, despite no significant change in spermidine blood levels, laboratory parameters or overall health status occured. No adverse events connected to spermidine intake were observed [LoE: *1b*].[123,124,a] In the same study by Schwarz *et al.*, a standardized repeated dose oral toxicity study was carried out in mice. In the tested animals, there were no significant changes of polyamine accumulation in several tissues, despite extremely high spermidine doses. Only in the highest dosing scheme (50 g / kg / day, for 28 days), spermidine was found to accumulate in cardiovascular tissue, but without detrimental effects. In fact, spermidine was shown to the risk of cardiovascular disease.[129] Another study confirmed the absence of adverse events in rats, using doses of 83 mg/kg/day.[141]

The preSmartAge study demonstrates that oral spermidine adminisitration is safe and well tolerated in mice and in older adults. The absence of adverse events also contributes to a good compliance rate of over 85%.[124] However, it raises questions regarding the oral bioavailability of spermidine and other polyamines. In humans as well as in mice, no significant changes in blood levels were observed. Polyamine

[a] ClinicalTrials.gov Identifier: NCT02755246

concentration in CNS tissue also showed no correlation to spermidine treatment and in mice, no changes in tissue concentrations of spermidine were observed.[124]

One possible explaination for the occurrence or non-occurrence of polyamine concentration changes in plasma could be the very fast absorption/metabolism rates of polyamines from the gastro-intestinal lumen into solid tissues. Various intestinal perfusion studies have shown a rapid absorption, as observed in ex vivo rat intestines [LoE: *in vitro*].[142] Spermidine can be detected in the portal vein within one minute after administration, yet the concentraation in systemic circulation still hardly exceeds 10-20 µM, as it is then rapidly absorbed into other compartments.[17,143] Another possible explaination is the short study duration in some trials. Soda *et al.* showed that after 6,5 months of oral polyamine supplementation, blood polyamine concetrations are up to 1,39-fold, as compared to volunteers that excluded polyamine-rich foods from their diet [LoE: *2b*].[144]

Regarding brain-blood-barrier (BBB) permeability of spermidine, early research of Shin *et al.* (1985) using radioactively marked ^{14}C polyamines showed that uptake in the brain is comparable to neurotransmitters as monoamines and acetylcholine (around 3-4%) [LoE: *in vivo*].[145] In pathological settings that involve a „leaky blood-brain barrier“ such as cerebral ischemia, intravenous injection of spermidine resulted in elevated levels of spermidine in brain parenchyma [LoE: *in vivo*].[17]

In respect to dosing, spermidine is taken up from the intestinal lumen by passive diffusion, and almost completely. The positive effects of memory function could be shown in doses of 1,2mg/day, which seems very low. Despite this apparent paradox of supply and low utilization, it has been repeatedly shown that orally administered polyamines are absorbed in sufficent amounts and used in corresponding tissues effectively.

6.4 Spermidine: Clinical potential

Regarding the clinical potential of spermidine, its direct AD-influencing capabilities are very promising, as spermidine supplementation has been proven to be beneficial

to counteract memory impairment in humans. Even if the pathways, that spermidine is interacting with, are not clearly laid out - especially in humans - the available evidence points in a very favorable direction. The challenge for future science may be to translate the *in vivo* knowhow, especially concerning pro-autophagic and anti-inflammatory properties, into human use. Spermidine does not qualify as a standalone treatment option for acute AD as the processes involved seem to be rather slow and low-dose dependant. Nevertheless, in recent years it became more and more apparent that low-dosed, long-term supplementation could be part of a strategy to prevent the formation of AD.

7 Conclusion

To sum up, this paper summarizes the available evidence of the three substances EGCG, fisetin and spermidine on the effects to counter AD in general, as well as some specific hallmarks of the disease. The findings and clinical potential, along with the ability of a given substance to counteract AD in general by improving memory function is summarized in **Table 5**.

Substance	**Clinical potential: AD in general / memory function**	**Clinical potential: Autophagy**	**Clinical potential: Neuro-inflammation**	**Clinical potential: Senescence**
EGCG	++ [*2b*]	++ [*in vitro*]	+++ [*in vivo*]	+ [*in vitro*]
Fisetin	+ [*in vivo*]	+ [*in vivo*]	+++ [*1b*]	+++ [*in vivo*]
Spermidine	+++ [*1b*]	++ [in vivo]	++ [*in vivo*]	? [*in vitro*]

Table 5: Summary of the clinical potential of the examined substances. Column 1 summarizes the evidence for the given substance to counteract AD and improve memory function as a whole.

The evidence reviewed in this paper implies a positive outlook for the analyzed substances. Whereas findings suggest that none of the substances is a viable option for AD monotherapy, a combination of substances could prove beneficial. Notably, all discussed compounds also show activities in scientific aspects of longevity, where AD is also a disease closely connected to processes of aging. In other age-related diseases, including heart disease, hypertension and cancer treatment, targeting more than one disease-feature is already state of the art.

Therefore, the author supports the concept that a combination therapy, as a mixture of pleiotropic (meaning to simultaneously produce more than one effect) natural compunds could emerge as a novel therapeutic method to successfully target neurodegenerative diseases, such as AD, in their early stage. In order to reach this

point, the definition of reliable biomarkers, as well as further studies on the discussed substances are necessary - especially in humans.

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