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Prevention of Cognitive Impairment in Subjects with Subjective Cognitive Decline APOE-4 Carriers after a Multimodal Lifestyle Intervention (PENSA Study): **The Role of the Endocannabinoid System**

BACHELOR THESIS
For obtaining the degree
Bachelor of Science (BSc.)
At the University for Veterinary Medicine Vienna

Submitted by
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Vienna, June 2023

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DECLARATION

Herby, I declare, that this thesis was solely written independently and has not been published elsewhere, in part or as whole. References and acknowledgments regarding citations are provided and summarised in an attached list.

Vienna, August 3rd, 2023

Ena Mašić

ACKNOLEDGEMENTS

I wish to express my gratitude to my primary supervisors Nieves Lozano Pizarro and Rafael de la Torre for giving me the opportunity of gaining first experience in being part of a clinical trial as well as providing guidance throughout my whole stay in Barcelona. It was of privilege, to work in such a friendly and welcoming laboratory. Nieves compassion and thoughtfulness supported me to keep a cool head even in times of crisis. Also, a big thank you to Mitona, who introduced me to the endocannabinoid method used in this thesis and Natàlia, who played an invaluable role in conducting the statistical analysis. I wish to extend my appreciation to my internal supervisor Rudolf Moldzio, for his great assistance throughout the whole process and for always keeping suggestions clear and direct.

Finally, I would like to express my deepest appreciation to my brother, parents and grandparents for the courage, love and moral support they keep providing for the goals I set for myself. Elea, Greta, Hugo, Ilvy, Iris and Viola, I thank you not only for your sincere friendship but also for making this bachelor´s study an unforgettable journey that I will cherish forever.

SUMMARY

Alzheimer`s disease (AD) is the most prevalent form of dementia. Currently there is no available treatment reversing pathological changes. Due to this, prevention of the disease is becoming increasingly important. Subjects with subjective cognitive decline (SCD), an early stage in the continuum of neurodegenerative disease progression, are at a higher risk for developing dementia and could therefore serve as a good target population for preventive intervention studies, such as the *"Prevention of cognitive decline in subjective cognitive decline APOE ε4 carriers after EGCG and a multimodal intervention"* study (PENSA study).

The PENSA study is built upon the premise of implementing lifestyle changes such as physical activity, cognitive training, social interaction and nutritional counselling combined with the dietary supplementation of epigallocatechin gallate (EGCG) to prevent or delay cognitive decline in SCD individuals carrying the *ε4* variant of the apolipoprotein-E (APOE) gene. So, individuals followed either 12 months of multimodal intervention (with EGCG or placebo) or regular lifestyle recommendations (control group).
In the context of the PENSA study, this thesis focuses on the critical role of the endocannabinoid system (ECS) in cognitive and memory processes that are compromised in AD and related diseases. Furthermore, as it is known that cardiovascular diseases (CVD) increase the risk of developing AD, it raises the prospect of both CVD and ECS being connected to the mechanisms underlying cognitive decline.

Consequently, the main hypothesis of the thesis is that the multimodal intervention can influence the ECSs activity, lower the risk for CVD and simultaneously improve cognition. To further investigate this connection and as a proxy of the brains ECS functionality, plasma ECs concentrations (N=129) have been analysed by UPLC-MS/MS at baseline, 6 and 12 months applying a previously validated methodology from our lab. Results show that the multimodal intervention appears to modulate both ECS and biomarkers of CVD. Thus, regarding the most relevant ECs, an increase in anandamide and decrease in 2-arachidonoylglycerol over 12 months was observed. No significant difference in concentrations related to sex could be established. Furthermore, the risk for CVD was reduced and the intervention had a favourable effect on cognition. Although improved cognitive performance cannot be directly attributed to changes in biomarkers for CVD and the ECS, a relationship between the components can be hypothesised.

ZUSAMMENFASSUNG

Die Alzheimer-Krankheit ist die am Weitesten verbreitete Form von Demenz. Zurzeit ist noch keine ursachenorientierte Behandlung vorhanden, wodurch die Prävention der Krankheit zunehmend an Bedeutung erlangt. In dieser Hinsicht sind Individuen mit subjektiver kognitiver Beeinträchtigung einem höheren Risiko für die Entwicklung von Alzheimer ausgesetzt und könnten dadurch eine gute Studienpopulation für präventive klinische Studien darstellen, wie z.B. die „Vorbeugung des kognitiven Abbaus bei APOE-ε4-Trägern mit subjektivem kognitivem Abbau (SCD) nach EGCG und einer multimodalen Intervention“ Studie (PENSA-Studie).

Die PENSA-Studie baut auf der Grundlage auf, dass Lifestyle Umstellungen, wie Erhöhung der physikalischen Aktivität, kognitives Training, soziale Interaktion und Ernährungsumstellung in Kombination mit einer Nahrungsergänzung mit Epigallocatechin Gallat (EGCG) in Individuen mit SCD und der genetischen Prädisposition der *ε4* Variante des Apolipoproteins-E (APOE), kognitive Abnahme vorbeugen oder drosseln können. Studienteilnehmer folgten 12 Monate lang entweder die multimodale Intervention (mit EGCG oder Placebo) oder reguläre Empfehlungen zur Lebenshaltung.
Im Kontext der PENSA-Studie, fokussiert sich diese Arbeit auf die Rolle des Endocannabinoid Systems (ECS) in kognitiven Prozessen, die bei Demenz beeinträchtigt sind. Da kardiovaskuläre Krankheiten (KVK) ebenso mit Alzheimer in Verbindung gesetzt worden sind, besteht die Möglichkeit, dass das ECS und KVK mit der Entwicklung von Alzheimer zusammenhängen könnten.

In dieser Arbeit wurde untersucht, ob eine multimodale Intervention die verteilte Menge an Endocannabinoiden (EC) beeinflussen, das Risiko für KVK erniedrigen und die kognitive Leistung steigern kann. Um diesen Zusammenhang zu prüfen, wurden Plasmaproben (N=129) mit Hilfe einer UPLC-MS/MS und einer zuvor validierten Methode unseres Labors, zu Beginn der Studie, 6 und 12 Monate danach analysiert. Die Ergebnisse deuten darauf hin, dass die Intervention das ECS und Biomarker der KVK moduliert. Angesichts der relevantesten EC, wurde eine Zunahme der Anandamid und Abnahme der 2-Arachidonoylglycerol Konzentration nach 12 Monaten nachgewiesen, wobei keine Unterschiede in Bezug auf das Geschlecht erkannt werden konnten. Obwohl die nachgewiesene verbesserte kognitive Leistung nicht direkt auf Veränderungen der Biomarker für CVD und das ECS zurückgeführt werden kann, lässt sich ein Zusammenhang zwischen den Komponenten annehmen.

ABBREVIATIONS

2-AG	2-arachidonoylglycerol
2-LG	2-linoleoyl glycerol
2-OG	2-oleoyl glycerol
AD	Alzheimer`s disease
AEA	N-arachidonoyl-ethanolamine; anandamide
APOE	apolipoprotein-E
APP	amyloid precursor protein
Aβ	beta-amyloid peptides
BDNF	brain-derived neurotrophic factor
CB1R	cannabinoid receptors 1
CB2R	cannabinoid receptors 2
CVD	cardiovascular diseases
DEA	N-docosatetraenoyl ethanolamide
DGLEA	N-dihomo-γ-linolenoyl ethanolamide
DHEA	N-docosahexaenoyl ethanolamide
ECS	endocannabinoid system
EGCG	epigallocatechin-3-gallate
ERC	endocannabinoid-related compounds
GDP	guanosindiphosphate
GPCR	G-protein coupled receptors
GTP	guanosintriphosphate
HOMA	homeostasis model assessment-estimated insulin resistance
ISTD	internal standard
LEA	N-linoleoyl ethanolamide
MedDiet	mediterranean diet
NCDs	non-communicable diseases
NFTs	neurofibrillary tangles
OEA	N-oleoyl ethanolamide
PA	physical activity
PEA	N-palmitoyl ethanolamide

POEA	N-palmitoleoyl ethanolamide
SCD	subjective cognitive decline
SEA	N-stearoyl ethanolamide
TyG	triglyceride-glucose
UPLC	ultra-performance liquid chromatography

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1 INTRODUCTION

1.1 Alzheimer's Disease (AD)

In 1907 Alois Alzheimer first presented his findings on the *"peculiar severe disease process of the cerebral cortex"* at a lecture in Tübingen. The changes in brain histology where later found out to be amyloid plaques and neurofibrillary tangles, which per definition count as one of the neuropathological hallmarks of Alzheimer's Disease (AD) (Hippius & Neundörfer, 2003; Serrano-Pozo et al., 2011). His findings remained unrecognized by the majority of scientist for almost 50 years (Hippius & Neundörfer, 2003).

To date, about 55 million people worldwide suffer from dementia, with AD being responsible for two thirds. Thereby making it the most frequent neurodegenerative disease responsible for dementia (Duong et al., 2017, Gautier S. et al. 2022). By 2050 the number of affected individuals is expected to reach 139 million people (Gauthier S. et al. 2022).

1.1.1 Definition and Classification

Dementia is the most severe form of neurological impairment and individuals suffering from dementia experience progressive cognitive decline (Duong et al., 2017; Jessen et al., 2020). The symptomatology is characterized by changes in functionality, cognition and behaviour and independent action is therefore impaired and limited. Dementia can be divided into four subtypes: AD, vascular dementia, Lewy body dementia and frontotemporal dementia (Duong et al., 2017). The most prevalent form is AD, which is responsible for 60-70 % of all cases (Fratiglioni et al., 2007). It is defined by a stealthy and slow progressing decline which starts with difficulties in retrieving short-term memory and gradually advances to troubles in executive functions (Duong et al., 2017). The latter incorporates activities that are involved in complex cognitive processes that mainly involve the prefrontal cortex, solving new problems or adapting one's behaviour to new information (Elliott, 2003). On a pathological level AD primarily known for two biomarkers, beta-amyloid plaques and neurofibrillary tangles. By inducing neuronal injuries, which evolve to neuronal death later on, neurotransmission is affected and can negatively influence cognition and memory (Duong et al., 2017). These two biomarkers are one of the main hallmarks of AD (Serrano-Pozo et al., 2011).

Beta-amyloid peptides (Aβ) derive from the amyloid precursor protein (APP). Disruptions regarding its synthesis can cause an excessive extracellular generation leading to accumulation and formation of plaques located in the isocortex (Gouras et al., 2014; Serrano-Pozo et al., 2011).
Neurofibrillary tangles (NFTs) on the other hand, are described by aggregated tau protein. Tau ordinarily stabilizes microtubules and thus promotes the axonal transport. Pathological hyperphosphorylation of tau can lead to aggregation and misfolding, forming NFTs. It could be observed that progression of cognitive decline in AD correlates with the piled-up amount of NFTs (Serrano-Pozo et al., 2011).

1.1.2 Subjective Cognitive Decline (SCD)

To date there have been no promising research outcomes for treating the underlying causes, beta-amyloid plaques and NFTs, of AD (Vaz & Silvestre, 2020). Furthermore physio-pathological changes can occur up to 20 years prior to the start of symptoms making it of high research interest to contain the onset of cognitive impairment as early as possible (Forcano et al., 2021).
As research discovered that Subjective cognitive decline (SCD) approximately occurs ten years before the official diagnosis takes place (Jessen et al., 2020), individuals with SCD present a good target group for preventative research studies on neurodegenerative diseases such as AD (Forcano et al., 2021). The Subjective Cognitive Decline Initiative (SCD-I) set a general terminology and framework to explore SCD in AD (Jessen et al., 2014). Leading to the general definition, that SCD refers to the individuals´ self-reported experience of increased or more frequent occurrences of confusion or memory loss. (Jessen et al., 2020)

1.1.3 Risk factors for developing AD

To successfully prevent or minimize the progress of cognitive decline trough preventative interventions it is important to understand the numerous risk factors involved in altering Aβ and tau aggregation. According to Armstrong (2019) the essential components can be divided into seven groups: demographic, genetic, lifestyle, medical, psychiatric, environmental and infection related. Thereby Alzheimer´s can be considered a multifactorial disorder, connected to an individual's overall health status. The main emphasis in this thesis will lay on following aspects: demographics; lifestyle factors; genetics focusing on

apolipoprotein E (APOE) and medical conditions focusing on cardiovascular disease (Armstrong, 2019).

Grouping	Risk factor
Demographic	Age [43,78,76]
	Education [76,92]
	Gender [76]
	Race [76]
	Social class [76]
Genetics	Amyloid precursor protein (*APP*) [39,58]*
	Presenilin 1 and 2 (*PSEN1/2*) [112,170]*
	Apolipoprotein E (*APOE*) [110,166,181]
	ATP-binding cassette transporter A1 (*ABCA1*) [105]
	Adaptor protein evolutionarily conserved signalling intermediate in Toll pathway (*ECSIT*) [175]
	Clusterin gene (*CLU*) [150]
	Estrogen receptor gene (*ESR*) [162]
	Fermitin family homolog 2 gene (*FERMT2*) [38]
	Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) [5]
	Histocompatibility locus antigen (HLA class III) [40,77,190]
	mtDNA haplotype [165]
	Transferrin gene (*Tf*) [194]
	Triggering receptor expressed on myeloid cells 2 (*TREM 2*) [161]
	Vascular protein sorting-10 domain (*VpS10*) genes [108]
	Vitamin D receptor gene (*VDR*) [193]
	Epigenetic factors [106,107]
Lifestyle	Alcohol [65]
	Lack of exercise [76,102,152]
	Lack of cognitive activity [92,174]

Grouping	Risk factor
	Malnutrition [1,76,189]
	Poor diet [65,167,168,176,196]
	Smoking [37,76,173]
Medical	Cancer [173]
	Cardiovascular disease [76,95,116]
	Congestive heart failure [76,81]
	Immune system dysfunction [12,128,141,158]
	Micro-infarcts [27]
	Obesity [31,71,125,185]
	Poor cholesterol homeostasis [110]
	Poorly controlled type-2 diabetes [4,118,124]
	Stroke [203]
	Traumatic brain injury (TBI) [53,76,80,93, 129,130,131,139,169]
Psychiatric	Depression [50,21]
	Early stress [82,148]
Environmental	Air pollution [101]
	Calcium deficiency [76]
	Geographic location [76]
	Metals (especially aluminium, copper, zinc) [16,86,147,156]
	Military service [60]
	Organic solvents [76]
	Occupation [101,147]
	Vitamin deficiency [68,126,139]
Infection	Bacteria, e.g. *Chlamydophila pneumonia*, *Treponema* [19,55,133]
	Dental infections [159]
	Fungi [7]
	Viruses [114,164,197]

Figure 1: Major risk factors connected to AD
Factors marked with a (*) are suggested to be a direct cause of AD (Armstrong, 2019).

Demographics

Regarding all demographic factors, age has the biggest impact on cognitive decline. AD risk is increased by 18 % with accelerating age and many of the pathological changes due to AD and age are only different in view of their severity. These include e.g. alterations in brain volume or loss of synapses. Another important factor is sex (Armstrong, 2019). Overall, 75 % of AD cases are individuals of the female sex. However, when examining this context, some

misconceptions can arise. For example, the female population generally reaches an older age compared to males and as age has a big influence in developing Alzheimer´s the number of females with AD is accordingly higher. Nevertheless, sex and gender differences could affect the onset of the disease but should not be generalized as underlying subfactors must be taken into consideration (Mielke, 2018).

Apolipoprotein E (APOE)

At a genetic level, scientists are still incapable of fully understanding the numerous Alzheimer's disease cases worldwide. Allelic variation in the apolipoprotein E (APOE) is the most prevailing genetic influence in developing AD (Armstrong, 2013).

The *APOE* gene codes for a protein, that is important in transporting cholesterol thereby being involved in maintaining brain lipid homeostasis regarding AD. There are three major allelic variants: ε2, ε3 and ε4. They vary in specific amino acid changes on positions 112 and 158 resulting in different structures. Respectively, these isoforms have a different preference for lipids and lipoproteins. There is a recognizable prevalence connected with APOE as risk for AD increases as follows: ε4 > ε3 > ε2 (Wood et al., 2021). Research has shown, that 50 % of AD cases can be described by carrying the ε4 variant. As studies have found out, that ε2 significantly lowers the risk for AD by 66 % (Wood et al., 2021), 95 % of the time the onset of the disease is considered due to variations in APOE genotype (Raber et al., 2004). To further support the relation between APOE and AD, a connection between the immunoreactivity of APOE and beta-amyloid plaques and neurofibrillary tangles was found (Namba et al., 1991).

Regarding the worldwide distribution of the APOE gene, the ε3 variant is the most widespread, ranging from 69-85 %. Followed by ε4 , which shows a distribution up to 25% with lowest occurrence in the Mediterranean and Asia. With a frequency of around 7.3 % the rarest genotype is APOE ε2 and investigations established, that in comparison to ε4/ε4 carriers, their risk for acquiring AD is lower by 99.6 % (Wood et al., 2021).

Cardiovascular disease

Besides genetic predispositions, there are also other factors assumed to be involved in advocating the development of AD, such as cardiovascular diseases (CVD) (Stampfer, 2006). CVD combines several diseases that affect the heart and blood vessels and according to the WHO,17.9 million people die annually due to CVD, making it the worldwide leading cause of death. An intact cardiovascular system is important for maintaining cognitive function, which raises the assumption of a possible connection between CVD and AD (Armstrong, 2019). The

Rotterdam study (Breteler et al., 1994) found out, that outcomes of cognitive tests were lower in individuals, that have a history of vascular events such as a stroke. Furthermore, the two diseases share many risk factors which brings into question whether cognitive decline could be slowed down by improving vascular health (Breteler et al., 1994).
To identify a possible risk for CVD, the triglyceride-glucose (TyG) index can be used as it calculates the possibility of insulin resistance, as diabetes increases the prevalence for CVD. In contrast to the commonly used homeostasis model assessment-estimated insulin resistance (HOMA) index, the TyG appears to be more accurate in evaluating insulin resistance in diabetic and non-diabetic individuals (Tao et al., 2022). The index can be calculated with *Ln[fasting triglycerides(mg/dL)×fasting glucose(mg/dL)/2]* (Jin et al., 2018)

Diet

Lifestyle changes also play an important role in disease development and in general, adapting a healthy diet decreases the risk for non-communicable diseases (NCDs) (Rosenberg et al., 2020). NCDs describe chronic diseases, that predominantly are non-infectious, like AD (Caron et al., 2020). Certain nutrients have been shown to decrease the risk for dementia, such as omega-3 polyunsaturated fatty acids or vitamin A, C and E (Rosenberg et al., 2020). Moreover, obesity is being connected to insulin resistance and AD (Lloret et al., 2019).

1.2 The Endocannabinoid System (ECS)

The ECS is a complex system responsible for multiple regulatory functions and has been identified to play a critical role in different neurodegenerative and neuropsychiatric diseases such as Alzheimer's disease; among others also Parkinson's disease, multiple sclerosis, and anxiety disorders (Bisogno et al., 2008; Rodríguez Fonseca et al., 2005). It´s deregulation has been described in multiple neurodegenerative diseases and it is assumed, that lifestyle changes could positively alter its concentrations (Charytoniuk et al., 2020; Pazos et al., 2004).

The discovery of the ECSs receptors for (-)-Δ^9-tetrahydrocannabinol (THC), the psychoactive compound of marijuana, dates to the early 1990s (Pazos et al., 2004). Since then, it has been increasingly targeted as a therapeutic approach to treat pathological conditions as well as Alzheimer's disease. (De Petrocellis et al., 2004). Overall, the ECS is comprised of two cannabinoid receptors, 1 (CB1R) and 2 (CB2R), their associated endogenous ligands (endocannabinoids), and respectively all enzymes involved in the degradation and synthesis

of these ligands (Gallego-Landin et al., 2021). The following two chapter serve to give an overview of most eminent characteristics of the ECS.

1.2.1 Receptors

CB1R and CB2R are both G-protein coupled receptors (GPCR) (Gallego-Landin et al., 2021). This receptor family is a part of membrane proteins and initiates a cellular response to neurotransmitters among other things. Generally, GPCRs share similar structures. Their single polypeptide chain is made of seven transmembrane segments.

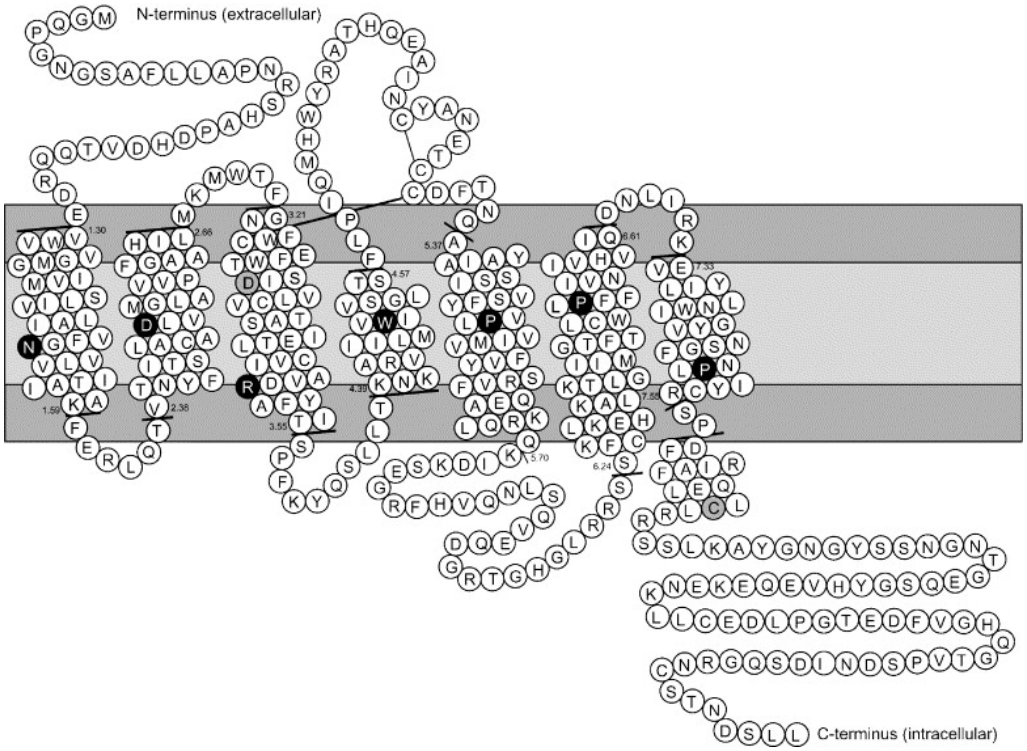


Figure 2: Structure of a G-protein coupled receptor
This figure illustrates the structure GPCRs with the example of the β-adrenoceptor. The seven transmembrane helices as well as the internal and external loops connecting them are visible (Kristiansen, 2004)

The associated G-Protein is composed of three subunits: α, β and γ. After activation through ligand binding the α subunit releases its previously bound guanosindiphosphate GDP, which then gets replaced with a guanosintriphosphate (GTP). This induces a conformational change that triggers the release of the G-protein and forces the α and βγ subunits to dissociate from the receptor, allowing them to further regulate signalling molecules (Alberts et al., 2017).

The first discovered receptor CB1R, is encoded by the gene *CNR1* and has a 472 long amino acid chain. So far two splice variants differing in a 33 amino acid deletion at the N-terminus have been identified (Zou & Kumar, 2018). Furthermore, CB1R is the most frequent GPCR in the brain. It is present in oligodendrocytes, astrocytes and microglia but has been found to be most expressed in neurons. The receptor is part of the neurotransmitter regulation and an implication with cell proliferation and death processes in the hippocampus is assumed, due to their involvement in activating the mitogen-activated protein kinase pathway (Gallego-Landin et al., 2021).
CB2R on the other hand is encoded by the gene *CNR2.* Its 360 amino acid long chain only shares 44 % homology with CB1R. For this endocannabinoid receptor also two isoforms are known (Zou & Kumar, 2018). They are expressed in immune cells of the central nervous system, such as macrophages, T and B cells and natural killer cells. After activation, the two receptors can be distinguished mainly by the fact that CB2R does not elicit psychotropic effects (Gallego-Landin et al., 2021).
Besides the main endocannabinoid receptors, it has been shown that ECs can also act as ligands for other receptors. An example is the Vanilloid VR1 receptor. It belongs to the transient receptor potential (TRP), which has been linked to modulation of pain (Chávez et al., 2010).

1.2.2 Endocannabinoids

The second component of the ECS are endocannabinoids (ECs) as they serve as ligands of the CBRs. The firsts discovered and most intensively researched ECs are N-arachidonoyl-ethanolamine (AEA; anandamide) and 2-arachidonoylglycerol (2-AG) (Zou & Kumar, 2018). Both derive from arachidonic acid and are conjugated with either glycerol (regarding 2-AG) or ethanolamine (regarding AEA). Their production is a response to the presynaptic Ca^{2+} release. Released postsynaptic 2-AG binds to CB1Rs positioned at the presynaptic terminal whereas AEA mostly remains within the synapse, targeting intracellular CB1Rs. The activation of presynaptic CB1Rs ultimately leads to inhibition of Ca^{2+} influx, resulting in a decrease of liberate neurotransmitters (Zou & Kumar, 2018).

Endocannabinoids are hydrophobic and uncharged, making them unable to diffuse through membranes as easily as neurotransmitters (Zou & Kumar, 2018). N-docosahexaenoyl ethanolamide (DHEA) is presumed to be another EC, because of managing to bind CB1R and CB2R with low affinity (Pastor et al., 2014). Moreover, there are endocannabinoid-related compounds (ERC) which share structural similarities with AEA and 2-AG but are different for

two reasons. They do not bind to endocannabinoid receptors nor are they containing highly unsaturated fatty acids but due to interaction with the same enzymes regarding synthesis and degradation it allows them to modify the ECS (Kleberg et al., 2014).

AEA. N-arachidonoyl-ethanolamine or also known as anandamide is a partial agonist for CB1R. (Zou & Kumar, 2018). In general agonists, lead to a full activation of the respective receptor and its subsequent signal cascades, whereas partial agonists only trigger partial efficacy of the receptor (von Forth et al., 2017). AEA also has been shown to activate the receptor TRPV1. As previously mentioned, anandamide release is triggered by elevated Ca^{2+} concentrations. Its synthesis is catalysed by N-acyl-phosphatidylethanolamine whereas the degradation is executed by fatty acid amide hydrolase. The product is free ethanolamine and arachidonic acid (Zou & Kumar, 2018).

2-AG. In contrast to AEA, 2-arachidonoylglycerol is a full agonist for both endocannabinoid receptors CB1R and CB2R. In most cases diacylglycerol lipase α generates 2-AG production from diacylglycerol. Regarding the initiation of synthesis, the same applies as with AEA. It is triggered by an increase of Ca^{2+}. Degradation proceeds by hydrolyzation through monoacylglycerol lipase into glycerol and arachidonic acid. Comparing brain concentration ratios, 2-AG has a 1000-fold higher basal level (Zou & Kumar, 2018).

Although research links the ECS to AD pathology (Bisogno et al., 2021; De Petrocellis et al., 2004), a high heterogeneity in EC concentrations throughout studies can be observed, possibly due to various applied methodologies (Berry et al., 2020). Charytoniuk et al. report the following context: downregulation of CB1R leads to a decrease in signalling; upregulation of CB1R showed neuroprotective outcomes and AD post-mortem studies exhibited elevated EC concentrations. Nevertheless, contradictory data exists, suggesting that further research is needed.

1.3 Multimodal Interventions

However, randomized clinical trials based on multidomain interventions, containing e.g., lifestyle changes and improvement of psychosocial factors, have been shown to have a positive effect on preventing cognitive decline (Rosenberg et al., 2020). Physical activity (PA)

and adapting a healthy diet not only have been successfully linked with AD pathology but also display an impact on EC concentrations (Armeli et al., 2021; Charytoniuk et al., 2020).

1.3.1 Physical activity

Even though PA modifies EC concentrations, study outcomes present bidirectional data, indicating that the ECS itself is a highly complex system. A possible way how PA could alter EC concentrations is by promoting the brain-derived neurotrophic factor (BDNF) (Charytoniuk et al., 2020). BDNF plays an important role in improving memory and cognition because of mediating short and long-lasting synaptic interactions, increasing neural and glial development and its neuroprotective properties (Kowiański et al., 2018). Subsequently it has a positive impact on brain plasticity. Neural progenitor cells have been shown to express a high amount of CB1R, further indicating a correlation between PA and the ECS (Tantimonaco et al., 2014).

1.3.2 Mediterranean diet

It was previously discussed that the ECS plays an important role in the glucose and lipid metabolism (Armeli et al., 2021) The deactivation of the CB1R resulted in reduced food intake, which led to the assumption that endocannabinoids alter food consumption (Armeli et al., 2021; Simon & Cota, 2017). Hence an overactivation of the ECS favours weight gain and insulin resistance, among others (Armeli et al., 2021). The Mediterranean diet (MedDiet) is composed as follows: avoidance of red meat; average consumption of dairy and fish; modest consumption of alcohol coupled with plant foods and olive oil as the main fat source. This adjustment has a positive influence on cognition and cardiovascular health (Lăcătușu et al., 2019).

1.3.3 Epigallocatechin-3-gallate (EGCG)

The bioactive phytochemicals produced by plants have proven to have a positive influence on health and prevention of disease (Yoo et al., 2018). A member of this chemical group is Epigallocatechin-3-gallate (EGCG) and its presence in green tea makes it easily available (Fareed & Chaudhary, 2022; Youn et al., 2022). It has been shown to have neuroprotective and anti-inflammatory effects due to its ability to regulate Aβ deposition and APP processing (Youn et al., 2022). This gives rise to clinical studies and research papers (Forcano et al., 2021; Sharifi-Rad et al., 2022; Wolfram, 2013) outlining the health benefits of EGCG.

With the goal of delaying subjective cognitive decline trough a multidomain approach multiple randomized clinical trials have been launched, such as the *"Prevention of cognitive decline in subjective cognitive decline APOE ε4 carriers after EGCG and a multimodal intervention"* (PENSA) study. This study is part of the world-wide Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability (FINGERS) network (Forcano et al., 2021). The FINGER initiative (ClinicalTrials.gov identifier number NCT01041989) was a 2-year long clinical trial to investigate the influence of multidomain interventions on cognitive impairment (Kivipelto et al., 2013).

1.4 Aims

The main aim of this thesis was to establish plasma endocannabinoid concentrations of the PENSA study participants (N=129) and subsequently conduct a longitudinal analysis for testing the influence of the study's multimodal intervention. Accordingly, the hypothesis of the thesis was, that over time a combination of improving mental health and physical activity coupled with a Mediterranean diet and EGCG supplementation can impact the distributed amount of endocannabinoids and consequently improve cognition, therewith leading to a decreased risk for developing AD. Additionally, the risk for cardiovascular disease calculated with the TyG index was explored. Likewise, sex differences were investigated. This raises the following questions:

i. Is the multimodal intervention capable of modifying endocannabinoid concentrations and are there differences between treatment arms (+/- EGCG)?
ii. How does the clinical trial impact cognition?
iii. Is the multimodal intervention displaying a positive effect on lowering cardiovascular risk?
iv. Are there any differences in terms of sex regarding the investigated properties?

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Equipment

-80 °C Freezer (Glacier -86°C Ultra-low Temperature Freezer, USA)

Centrifuge (Meditronic BL-S, J.P. Selecta, Spain)

Centrifuge (Multifuge 3L-R Refrigerated Centrifuge, Thermo Electron, USA and Heraeus, Germany)

Crimp/Snap Top Vials 2 mL (Agilent Technologies, USA)

Evaporator (TurboVap® LV, Caliper Life Science, USA)

Glass Pasteur Pipettes 150 mm (Delta Labs, Spain)

Glass Tubes (KIMAX® Reusable Screw Thread Culture Tube with cap, DWK Life Science, Germany)

H_2O Milli-Q Dispenser System (Milli-Q® Advantage A10, Merck Millipore, USA)

Liquid Chromatography Instrument (ACQUITY Premier System, Waters™, USA)

Mass Spectrometry Instrument (Xevo TQ-S micro, Waters™, USA)

pH meter (Basic 20, Crison, Spain)

Pipette tips (Capillary Pistons, Gilson, Germany)

Pipette tips (Daslab®, Spain)

Piston Pipette 1000 µL (PIPETMAN®, Gilson, Germany)

Positive Displacement Pipette 50 µL (MICROMAN®, Gilson, Germany)

Variable Repetitive Syringe Dispenser (Nichimate Stepper, Nichiryo, Japan)

Vial Caps 2 mL (Crimp/Snap Caps, Agilent Technologies, USA)

Vial Inserts (Agilent Technologies, USA)

Volumetric Flask 500 mL, 250 mL (Afora, Spain)

Vortex (Buchler REAX 2000 Vortex, Heidolph Instruments, Germany)

2.1.2 Chemicals

Acetic Acid 100 % (Merck, Germany)

Acetonitrile ≥ 99 % purity (ACN) (Honeywell, Riedel-de Haën™, Germany)

Ammonium Acetate 100 % (Merck, Germany)

Ammonium Acetate Buffer 0.1 M pH=4 (prepared by lab)

Ultrapure deionized Water (Milli-Q® Advantage A10, Merck Millipore, USA)

Formic Acid 98 %-100 % purity (Sigma Aldrich, Germany)

Internal Standard (ISTD)

2-AG-d5, AEA-d4, DHEA-d4, LEA-d4, PEA-d4, OEA-d4, SEA-d3 > 98 % (Cayman Chemical, USA)

2-OG-d5 > 94 % (Toronto Research Chemicals, USA)

Tert-butyl-methyl-ether for analysis, ACS (PanReac AppliChem ITW Reagents, Germany)

2.2 Methods

2.2.1 PENSA Study

The PENSA study is a 12-month randomized double blind clinical trial. A follow up three months after study termination is provided to investigate long term effects after the intervention. The study focuses on assessing the influence of a multimodal lifestyle intervention paired with epigallocatechin-3-gallate supplementation on cognitive performance (Forcano et al., 2021) The multimodal intervention included: (i) an individually adapted MedDiet, (ii) improving PA, (iii) cognitive training and (iv) group sessions for social stimulation (Forcano et al., 2021). The EGCG supplement consisted of 266-532 mg/day sachets intended for dissolution in 100 mL of water (de la Torre Fornell et al., 2020).

Study individuals represent a 60–80-year-old male and female population (N=129) carrying the APOE ε4 genotype in combination with meeting the subjective cognitive decline (SCD)

criteria. Thus, they are at higher risk for developing Alzheimer's Disease. After eligibility is met with these criteria, baseline cognition is evaluated (de la Torre Fornell et al., 2020). After these assessments only individuals with cognitive function within the norm and no visible abnormalities on neuroimaging scans are qualified for the study (Forcano et al., 2021).

The population was divided into three treatment arms. Two of them partake in the multimodal intervention and personal nutritional program supplemented with either EGCG or a placebo. The third group is only advised with lifestyle recommendations but does not follow a personalized program. As the study is double blinded and so far, not finished, the blind is currently still protected and treatment arms are referred to as A, B and the control group C (de la Torre Fornell et al., 2020).

The clinical trial can officially be found under the identifier number NCT03978052 on ClinicalTrials.gov. The study protocol maintains the standards of WAMA Declaration of Helsinki (Brazil, October 2013), European Union standards for clinical trials and good scientific practice (Directives 2001/20/EC and 2005/28/EC), and the General Data Protection Regulation (GDPR UE 2016/976). Furthermore, it is approved by the local institutional review board Parc de Salut Mar Clinical Research Ethics Committee (CEIm-PSMAR) (Forcano et al., 2021).

Variables

Sociodemographic and lifestyle information from participants at baseline (0 months) are evaluated: Age, APOE genotype, marital, work and smoking status, body mass index (BMI) and presence of a diabetes disease (yes/no). BMI cut off points are as follows: underweight BMI < 18.5 kg/m^2; normal weight BMI 18.5 – 24.9 kg/m^2; overweight BMI 25 – 29.9 kg/m^2 and obese BMI > 30 kg/m^2 (Weir & Jan, 2019).

Furthermore, cognition is measured at baseline, 6 and 12 months. For this following cognitive function assessment tests implemented in the clinical trial will be used: ADCS-PACC, ADCS-PACC-Plus-exe and MOCA. The Alzheimer's Disease Cooperative Study Preclinical Alzheimer Cognitive Composite (ADCS-PACC) is used to evaluate first signs of cognitive decline to prematurely diagnose AD. It involves tests checking for episodic memory, timed executive function and global cognition (Donohue et al., 2014).

Adding scores from the Five Digit test and the Stroop Color and World Test, results in the ADCS-PACC-Plus exe score, which also is being analysed (Forcano et al., 2021). Both are

standardized by using a z-score, which provides information on the number of standard deviations above or below the populations mean raw score. It is calculated with the formula Z = (x-M)/SD. X represents the original score, M the mean and SD the standard deviation (Andrade, 2021).

The Montreal Cognitive Assessment (MOCA) is a one-page test with a duration of 10 minutes. It checks for short-term memory recall, visuospatial abilities, executive functions, attention, concentration, working memory, language and orientation to time and place. An overall of 30 points can be achieved (Nasreddine et al., 2005). Scores above 26 represent “normal” cognitive abilities (Thomann et al., 2018).

Data of variables described above was obtained prior to this work and was provided to conduct subsequent analysis and correlation with TyG and EC concentrations.

2.2.2 Preparation of Internal Standard

Instead of a calibration curve an internal standard (ISTD) mixture containing deuterated forms of the most relevant ECs was chosen for comparison of quantified compounds. Deuterated analogues of the endocannabinoids were relevant to differentiate between them and their related compound by mass spectrometry in subsequent analysis. The commercially solid ISTD components were dissolved in acetonitrile (ACN) (Honeywell, Riedel-de Haën™, Germany) to reach following final concentrations: 0.01 µg/mL AEA-d4 and DHEA-d4, 0.02 µg/mL LEA-d4. 0.04 µg/mL PEA-d4, OEA-d4 and SEA-d3, 0.2 µg/mL 2-AG-d5 and 1 µg/mL2-OG-d5.

Since some compounds do not have a commercially available deuterated ISTD, analogous standards with similar molecular structures were used for calculation of their concentration. This applied for 2-LG, DEA, DGLEA and POEA.

The internal standard was stored at -23 °C until further use and in all cases, quantification was performed by comparing areas of each target compound with a known concentration of their corresponding ISTD.

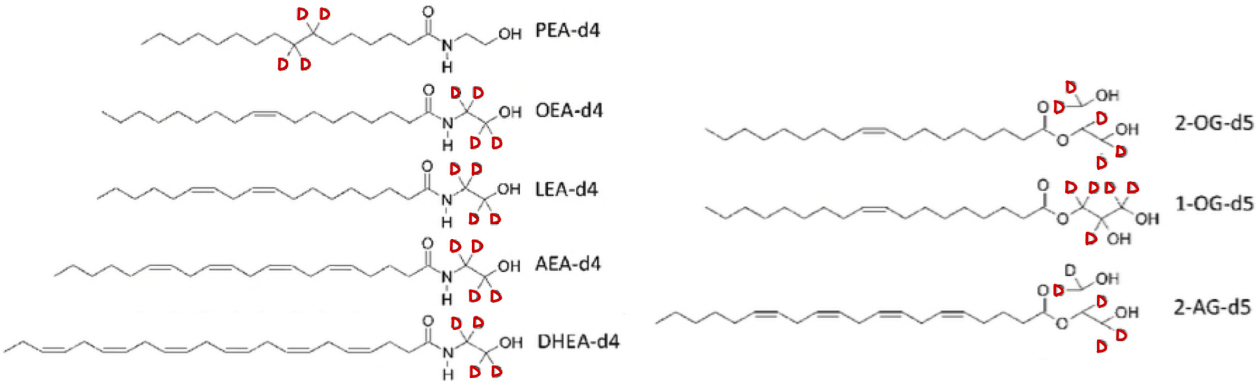


Figure 3: Structure of deuterated ECs and ERCs
Deuterated analogues deviate from their related compound by a substitution of hydrogen with deuterium. The difference in structure is marked in red (Pastor et al., 2014).

2.2.3 Sample Preparation for Endocannabinoid Quantification

The following sample preparation was experimentally investigated and published by Pastor et al. (2014). Plasma samples were collected at baseline (0 months), 6 months and 12 months from every study participant and stored at -80 °C until further procedures. Maintenance of sample cold chain was of crucial importance as ex vivo formation and chemical isomerization of 2-monoacylglycerols could alter results.

Ammonium Acetate Buffer

Ammonium acetate buffer, 0.1 M, pH=4 was required for liquid-liquid extraction. For the buffer preparation 1.93 g ammonium acetate (Merck, Germany) was dissolved in 200 mL H_2O Milli-Q water (Merck Millipore, USA) in a volumetric flask (Afora, Spain) of 250 mL capacity. Acetic acid (Merck, Germany) was used to adjust the buffer to a pH 4 and additionally the flask was filled up to 250 mL to reach a final concentration of 0.1 M. The pH was measured with the Basic 20 pH meter (Crison, Spain) and the finished buffer was stored at -23 °C until further use.

Plasma Preparation

For the experiment, plasma samples were defrosted to room temperature and subsequently put on ice to prevent an increase of endocannabinoid concentrations. 500 µL of the sample were transferred to a KIMAX® glass tube (DWK Life Science, Germany). Deviations of this volume were noted to accordingly adapt calculation of concentrations to achieve a correct

generation of results. Next 500 µL of ammonium acetate buffer and 25 µL of ISTD containing deuterated compounds were added. The liquid-liquid-extraction was performed by adding 5 mL of tert-butyl-methyl-ether (PanReac AppliChem ITW Reagents, Germany) and placing the tubes in the rocking tube mixer for 20 minutes. This step served to extract the compounds from the aqueous to the organic phase. The tubes were then centrifuged at 2711 g for 5 minutes at 4 °C (Multifuge 3L-R Refrigerated Centrifuge, Thermo Electron, USA and Heraeus, Germany). After separating the organic from the aqueous phase with a glass Pasteur pipette (150 mm, Delta Labs, Spain) and collecting it in a new clean tube, the organic phase was evaporated to dryness at ≤ 39 °C and a gentle nitrogen pressure of ≤ 15 psi using the TurboVap® LV Evaporator (Caliper Life Science, USA).

Reconstitution was carried out by adding 150 µL 90:10 acetonitrile/formic acid solution and vortexing the tubes for five seconds with Buchler REAX 2000 Vortex (Heidolph Instruments, Germany). Finally, the samples were transferred to a 2 mL vial (Agilent Technologies, USA) containing an insert vial (Agilent Technologies, USA). Until LC/MS-MS analysis the samples were stored at 4 °C.

For each analytical batch a blank of reagents without the ISTD, that was chemically treated as the samples was included. The blank is necessary to monitorize eventual interferences during the chromatography separation and possible contaminations during the sample preparation.

2.2.4 Liquid Chromatography – Tandem Mass Spectrometry (LC/MS-MS)

The following ECs and related compounds where quantified: 2-arachidonoyl glycerol (2-AG), 2-linoleoyl glycerol (2-LG), 2-oleoyl glycerol (2-OG), N-arachidonoyl ethanolamide (AEA; anandamide), N-docosatetraenoyl ethanolamide (DEA), N-dihomo-γ-linolenoyl ethanolamide (DGLEA), N-docosahexaenoyl ethanolamide (DHEA), N-linoleoyl ethanolamide (LEA), N-oleoyl ethanolamide (OEA), N-palmitoyl ethanolamide (PEA), N-palmitoleoyl ethanolamide (POEA) and N-stearoyl ethanolamide (SEA).

Table 1: Mass-to-charge ratio of parent and daughter ion for all quantified compounds

Compound name	*Parent (m/z)*	*Daughter (m/z)*
2-AG	379	287
2-LG	355	263
2-OG	357	265
AEA	348	62
DEA	376	62
DGLEA	350	62
DHEA	372	62
LEA	324	62
OEA	326	62
PEA	300	62
POEA	298	62
SEA	328	62

Ultra-performance liquid chromatography (UPLC) was performed using an Acquity Premier liquid chromatography device (Waters™, USA) For the reverse phase separation an Acquity UPLC® BEH C18 1.7 µm column (2.1 x 100mm column, Waters™, USA) was chosen. The sample manager preserved a temperature of 4 °C while the chromatographic separation was carried out at 50 °C.

The mobile phases A and B were equally prepared, resulting in and 0.01 % (v/v) formic acid in acetonitrile (A) representing the organic phase and 0.01 % (v/v) formic acid in Milli-Q water (B) representing the aqueous phase. The flow rate was 0.4 mL/min and the solvent ratio was composed as follows: The initial ratio of 40 % A was maintained for the first two minutes. Then A was increased to 65 % at the 3rd min, to 80 % at the 10th min leading to the highest concentration of 100 % at the 11th min. Subsequently the initial gradient ratio was restored at minute 14.1. Resulting in an overall duration of 15.5 min per run.

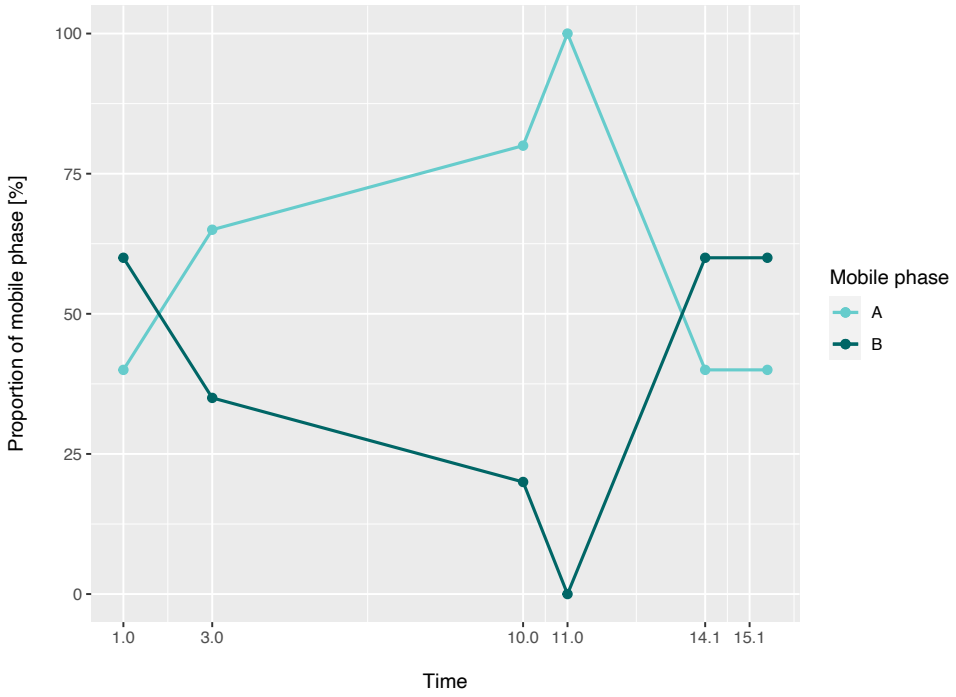
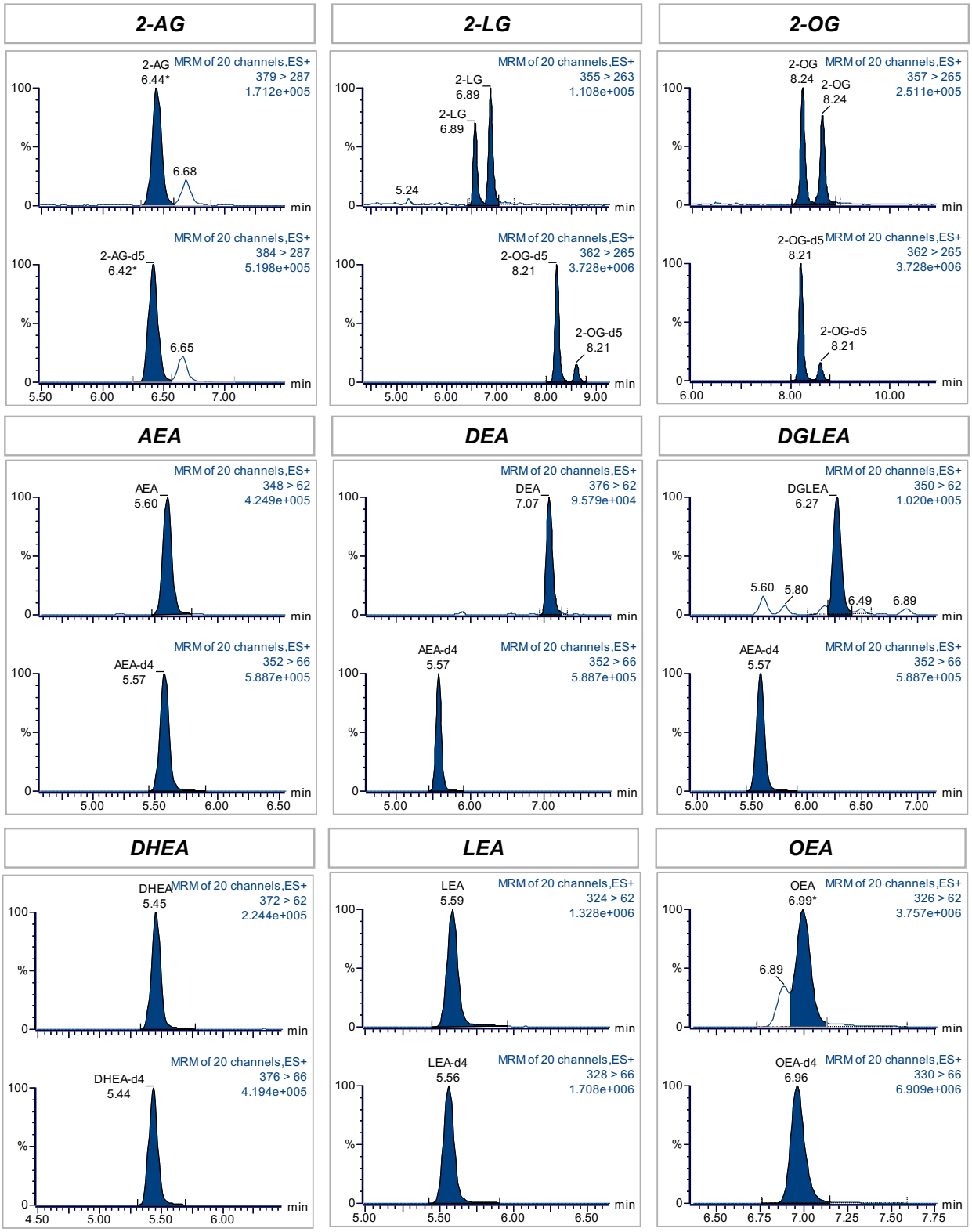


Figure 4: Gradient elution and solvent ratio composition of HPLC run
Graph displays solvent ratio during one run. Each line represents one mobile phase. (A) representing the organic and (B) representing the aqueous phase.

For the tandem mass spectrometry measurement, the UPLC instrument was coupled with a Xevo Triple Quadrupole mass spectrometry device (Waters™, USA). Each analyte was detected by multiple reaction monitoring and a Zspray™ for positive electrospray ionization. The cone voltage for the electrospray was set to 20 V for all compounds except for SEA with 15 V. The desolvation gas nitrogen was used at 600 °C and with a flow rate of 1,200 L/hr. Different collision energy values for fragmentation of compounds were set for ECs and ERCs. For 2-AG, 2-AG-d5, 2-LG, 2-OG, 2-OG-d5 collision energy was set to 15 V whereas for the remaining compounds 20 V where necessary.

Results were acquired and managed with MassLynx™ software. Additionally, chromatograms were processed and integrated with Target-Lynx™ Application Manager. Integration has been done individually for each compound in every sample.


2-AG
2-AG 6.44*
6.68
MRM of 20 channels,ES+ 379 > 287 1.712e+005
2-AG-d5 6.42*
6.65
MRM of 20 channels,ES+ 384 > 287 5.198e+005
2-LG
2-LG 6.89
2-LG 6.89
5.24
MRM of 20 channels,ES+ 355 > 263 1.108e+005
2-OG-d5 8.21
2-OG-d5 8.21
MRM of 20 channels,ES+ 362 > 265 3.728e+006
2-OG
2-OG 8.24
2-OG 8.24
MRM of 20 channels,ES+ 357 > 265 2.511e+005
2-OG-d5 8.21
2-OG-d5 8.21
MRM of 20 channels,ES+ 362 > 265 3.728e+006
AEA
AEA 5.60
MRM of 20 channels,ES+ 348 > 62 4.249e+005
AEA-d4 5.57
MRM of 20 channels,ES+ 352 > 66 5.887e+005
DEA
DEA 7.07
MRM of 20 channels,ES+ 376 > 62 9.579e+004
AEA-d4 5.57
MRM of 20 channels,ES+ 352 > 66 5.887e+005
DGLEA
DGLEA 6.27
5.60
5.80
6.49
6.89
MRM of 20 channels,ES+ 350 > 62 1.020e+005
AEA-d4 5.57
MRM of 20 channels,ES+ 352 > 66 5.887e+005
DHEA
DHEA 5.45
MRM of 20 channels,ES+ 372 > 62 2.244e+005
DHEA-d4 5.44
MRM of 20 channels,ES+ 376 > 66 4.194e+005
LEA
LEA 5.59
MRM of 20 channels,ES+ 324 > 62 1.328e+006
LEA-d4 5.56
MRM of 20 channels,ES+ 328 > 66 1.708e+006
OEA
OEA 6.99*
6.89
MRM of 20 channels,ES+ 326 > 62 3.757e+006
OEA-d4 6.96
MRM of 20 channels,ES+ 330 > 66 6.909e+006

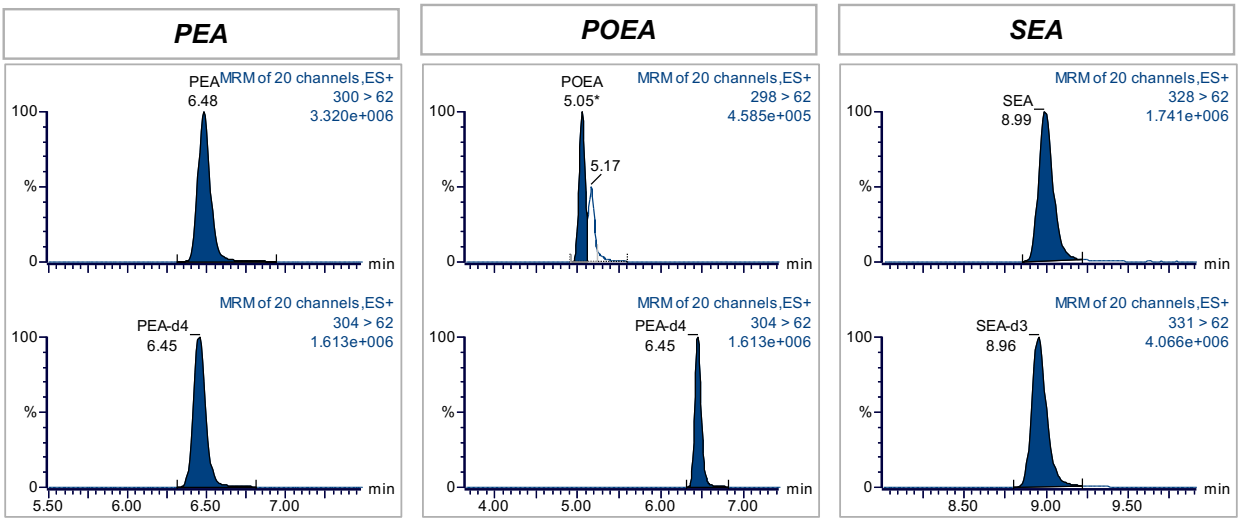


Figure 5: Overview of peak integration for all compounds
For each EC and ERC compound and its respective ISTD peak are shown. The y-axis represents the peaks intensity, whereas the x-axis stands for the retention time in minutes. “MRM of 20 channels, ES+” indicates the type of scan. In this case Multiple Reaction Monitoring (MRM) and a positive ionization are used as the mass

Afterwards compound concentrations were obtained with following formula:

$$[endocannabinoid]\ \frac{ng}{mL} = \frac{ISTD\ in\ ng\ x\ anaylte\ response}{ISTD\ response\ x\ RT\ x\ aliquot\ volume\ in\ mL}$$

The ratio of the response area of the analyte to response area of the internal standard (ISTD) represents the response factor (RT).

2.2.5 Statistical Analysis

Participants were chosen by the previously mentioned criteria. Raw results were obtained with UPLC, concentrations calculated with Excel and statistical analysis as well as creation of graphs and table was performed with R-Studio. Significance was tested by applying one-way ANOVA, linear mixed-effect models or a Welch two sample t-test. In case of demographic description, a Chi-test for categorical values was used. Data was considered statistically significant if the p-value remained under 0.05. Clarification about the test used is given under each graph individually.

3 RESULTS

For analysis of baseline (0 months) EC concentrations, all treatment groups are included. Analysis of longitudinal change of plasmatic endocannabinoid concentrations was performed in two ways. For sex comparison the control group is excluded from the database to investigate if the multimodal intervention has a different impact on each sex. Regarding treatment investigation, intervention groups (+/-EGCG) were grouped together as clinical trial is still blinded and no current information on supplementation with EGCG/placebo is available. Thus, intervention group is aligned against the control group C to test if the study design has an impact on EC concentrations.

3.1 Demographic description of the study population at baseline (0 months)

Demographic description of the study population at baseline is described in **table 1**. Briefly, the overall study participant number was 129, 64 females and 45 males, with an average age of 67.2 years. All of them are carriers of the APOE ε4 genotype, met SCD criteria and had cognitive outcomes associated with healthy cognition. APOE distribution was 6.98 % heterozygotes ε2/ε4, 86.00 % heterozygotes ε3/ε4 and 6.98 % homozygotes ε4/ε4. The population were divided in three study arms: two treatment arms (A and B) containing 52 participants each and a control group with 25 participants. Mean number of years of education was 9.88 years and the majority was married or had a common law partner (82.90 %). Regarding work status 15.5 % were employed, 80.6 % were retired and the remaining 3.88% were unemployed. Smoking status was divided in current or former smoker (58.1 %) and individuals who never smoked (41.9 %). BMI calculation yielded the following results: 45 % with normal weight, 17.6 % obese, 32 % overweight and a minority of 4 % underweight. In terms of diabetes, most individuals (88.2 %) reported not having this medical disease.

Table 2: Demographic overview of the study population at baseline

	All N=129	***Female*** N=84	***Male*** N=45	***P-value***
		h (p %)		
Age	67.2 (4.82)	67.4 (4.79)	66.8 (4.89)	0.455
APOE Genotype				1.000
2/4	9 (6.98 %)	6 (7.14 %)	3 (6.67 %)	
3/4	111 (86.0 %)	72 (85.7 %)	39 (86.7 %)	
4/4	9 (6.98 %)	6 (7.14 %)	3 (6.67 %)	
Treatment Arm				0.691
A (EGCG or placebo)	52 (40.3 %)	36 (42.9 %)	16 (35.6 %)	
B (EGCG or placebo)	52 (40.3 %)	33 (39.3 %)	19 (42.2 %)	
C (Control Group)	25 (19.4 %)	15 (17.9 %)	10 (22.2 %)	
Education	9.88 (3.33)	9.98 (3.39)	9.71 (3.26)	0.665
Marital Status				**0.005**
Married or common law partner	92 (82.9 %)	53 (74.6 %)	39 (97.5 %)	
Single	10 (9.01 %)	9 (12.7 %)	1 (2.50 %)	
Widowed	9 (8.11 %)	9 (12.7 %)	0 (0.00 %)	
Work Status				0.302
Employed	20 (15.5 %)	10 (11.9 %)	10 (22.2 %)	
Retired	104 (80.6 %)	70 (83.3 %)	34 (75.6 %)	
Unemployed	5 (3.88 %)	4 (4.76 %)	1 (2.22 %)	
Smoking Status				0.381
Current or former smoker	75 (58.1 %)	46 (54.8 %)	29 (64.4 %)	
Never smoked	54 (41.9 %)	38 (45.2 %)	16 (35.6 %)	
BMI				**<0.001**
Normal weight	57 (45.6 %)	47 (58.0 %)	10 (22.7 %)	
Obese	22 (17.6 %)	10 (12.3 %)	12 (27.3 %)	
Overweight	41 (32.8 %)	20 (24.7 %)	21 (47.7 %)	
Underweight	5 (4.00 %)	4 (4.94 %)	1 (2.27 %)	
Diabetes				0.053
No	90 (88.2 %)	64 (92.8 %)	26 (78.8 %)	
Yes	12 (11.8 %)	5 (7.25 %)	7 (21.2 %)	
ADCS-PACC	0.00 (0.66)	0.02 (0.66)	-0.03 (0.67)	0.711
ADCS-PACC-Plus-exe	0.00 (0.61)	-0.02 (0.62)	0.03 (0.60)	0.692
MOCA	26.6 (2.45)	26.8 (2.43)	26.3 (2.49)	0.310

h represents the respective overall participant number whereas p % represents the percentage share. Education is calculated in years. Cognitive assessment scores represent baseline results. ADCS-PACC and ADCS-PACC-Plus-exe mean outcomes are displayed as z-scores and MOCA as the overall mean scored at testing. Statistical significance between sex was tested with Students-t-test for numerical and Chi-squared test for categorical values ($p<0.05$). Significance is displayed in bold.

3.2 APOE genotype regarding EC concentrations at baseline

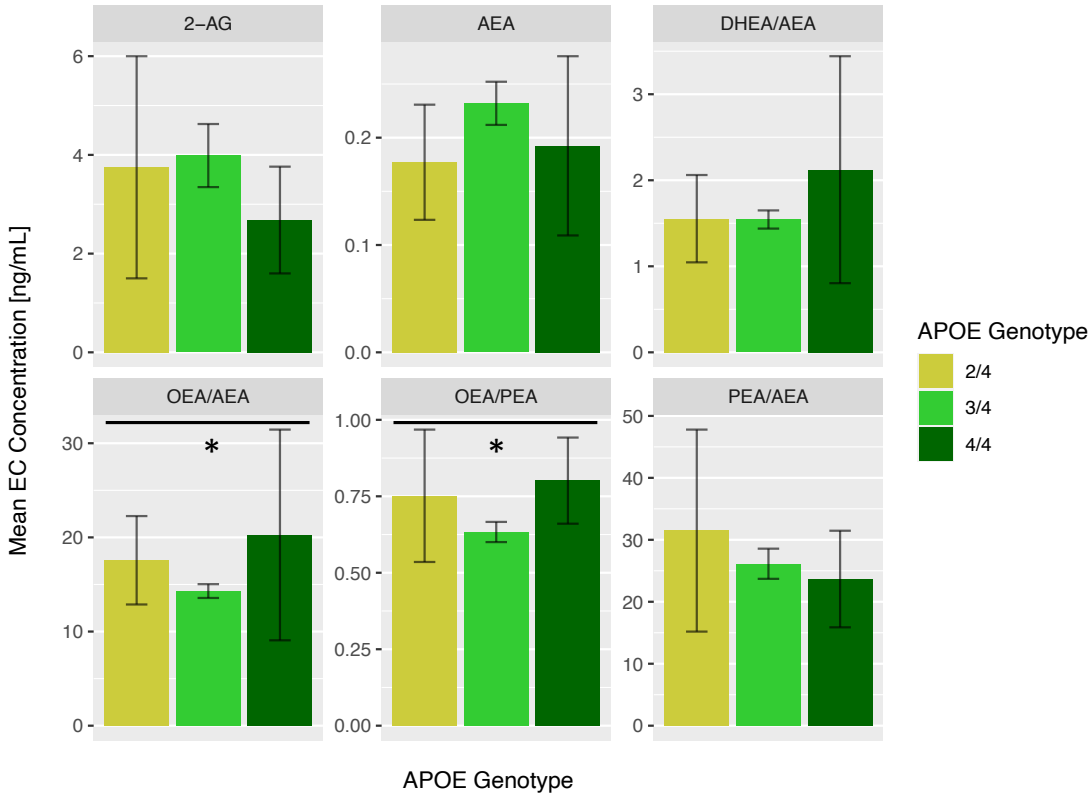


Figure 6: Baseline EC concentrations divided by APOE genotype
Bar chart displaying the mean baseline plasmatic endocannabinoid concentrations [ng/mL] divided by APOE genotype. Error bars display standard deviation and a confidence interval of 95 % was chosen.
Statistical significance was tested with an ANOVA ($p<0.05$). Statistically significant results are displayed with (*).

A high heterogeneity in distribution of concentrations was detected in relation to the APOE genotype **(Figure 6)**.

No clear trend can be derived from the graph. Differences in baseline EC concentration regarding APOE genotype only show significant difference in the EC ratio OEA/AEA ($p=0.0038$) and the OEA/PEA ($p=0.0111$) **(Table 2)**.

Table 3: P-values of baseline EC concentrations divided by APOE genotype

Compound	***2-AG***	***AEA***	***DHEA/AEA***	***OEA/AEA***	***OEA/PEA***	***PEA/AEA***
P-value	0.515	0.205	0.065	0.0038	0.0111	0.433

3.3 Sex differences in EC concentrations

3.3.1 Baseline analysis of sex differences

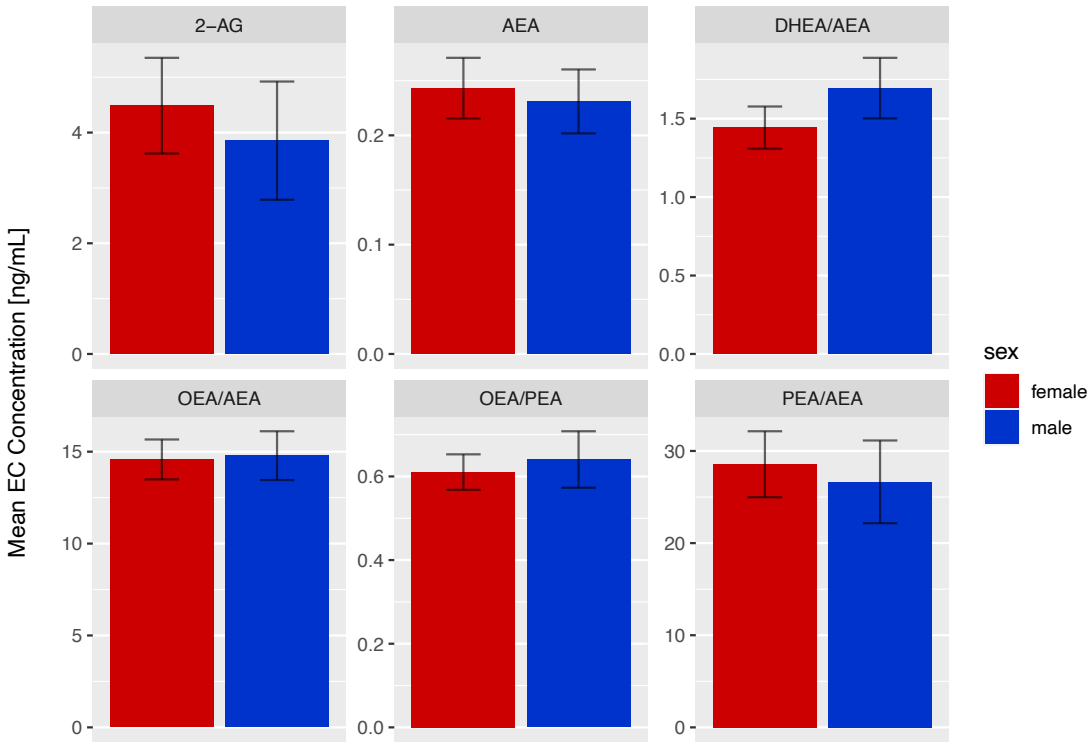


Figure 7: Baseline EC concentrations divided by sex
Bars display respective mean plasmatic endocannabinoid concentrations [ng/ml] grouped by female (red) and male (blue) at baseline. Additionally a confidence interval of 95 % was chosen and the error bars display the standard deviation of the mean from all the females and males.
Statistical significance was tested by applying a Welch two smaple t-test ($p<0.05$) to compare numerical variables. Significant values would have been shown with (*).

The sex differences of baseline EC concentrations and their corresponding ratios are shown in **figure 7**. In the case of 2-AG, DHEA/AEA and PEA/AEA a difference depending on sex could be assumed. However, analysing the baseline endocannabinoid concentrations grouped by sex did not show any statistical significance **(Table 3)**.

Table 4: P-values of baseline EC concentrations divided by sex

Compound	***2-AG***	***AEA***	***DHEA/AEA***	***OEA/AEA***	***PEA/AEA***	***OEA/PEA***
P-value	0.7824	0.5845	0.4521	0.4503	0.2959	0.6077

3.3.2 Longitudinal analysis of sex differences

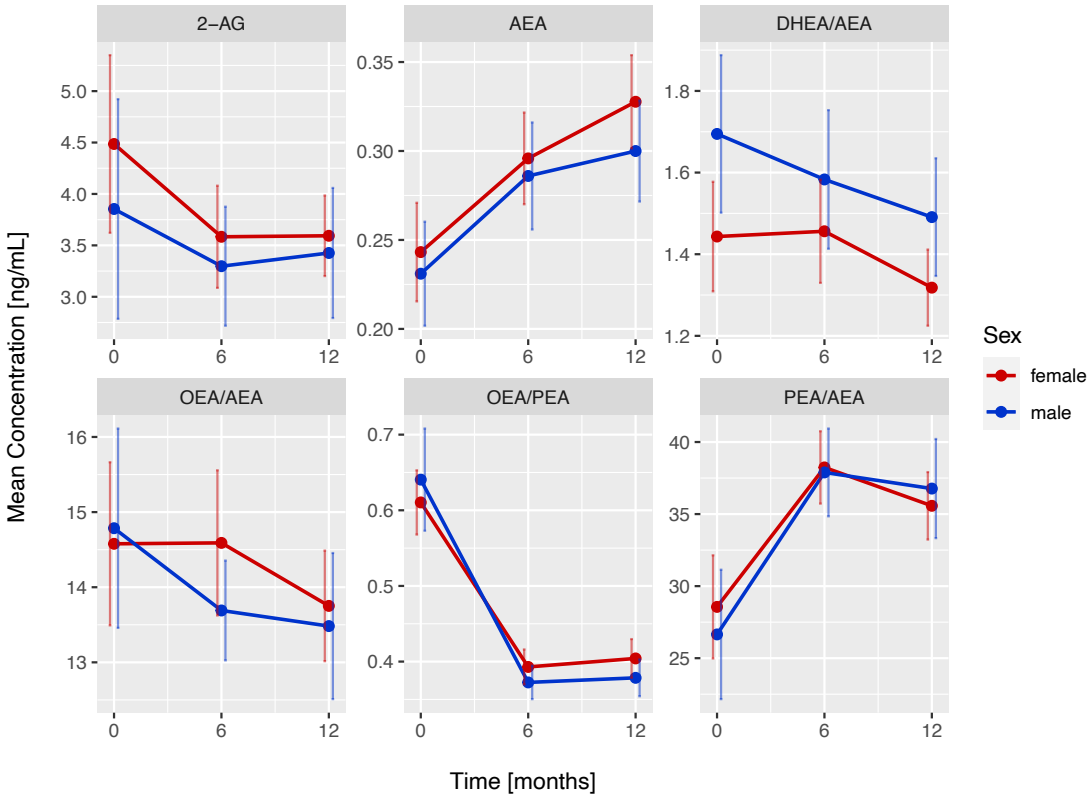


Figure 8: Change in EC concentrations over time grouped by sex
Plot displays the mean plasmatic endocannabinoid concentrations [ng/mL] at each time stamp (0, 6 and 12 months) with exclusion of participants administered to the control group. Error bars display standard deviation and a confidence interval of 95 % was chosen.
Statistical significance after 6 and 12 months compared to baseline was tested with a linear mixed-effects model ($p<0.05$). Statistically significant results would have been displayed with (*).

In relation to starting point (0-months) and end point (12-months) both the male and female sex showed the same tendencies in EC concentrations when all substances are considered individually **(Figure 8)**. In the case of 2-AG and AEA women always displayed a higher concentration of ECs (0, 6 and 12 months). In contrast to this, men displayed higher concentrations when looking at the ratio DHEA/AEA. For OEA/AEA and OEA/PEA men show higher baseline concentrations but after 6- and 12-months female concentrations increase and exceed them. For the ratio PEA/AEA the opposite was observed, apart from concentrations aligning at 6-months. The statistical test compares if there is a significant difference in concentration development between 0-6- and 0-12-months considering sex. Calculation showed no statistical significance **(Table 4)**.

Table 5: P-values of longitudinal EC concentrations divided by sex

Compound	*2-AG*	*AEA*	*DHEA/AEA*	*OEA/AEA*	*PEA/AEA*	*OEA/PEA*
P-value 0-6	0.9431	0.9335	0.7385	0.5439	0.7347	0.2744
P-value 0-12	0.8902	0.7709	0.2732	0.6574	0.5635	0.3390

3.4 Influence of multimodal intervention on EC concentrations

3.4.1 Longitudinal analysis of comparison between treatment group and controls

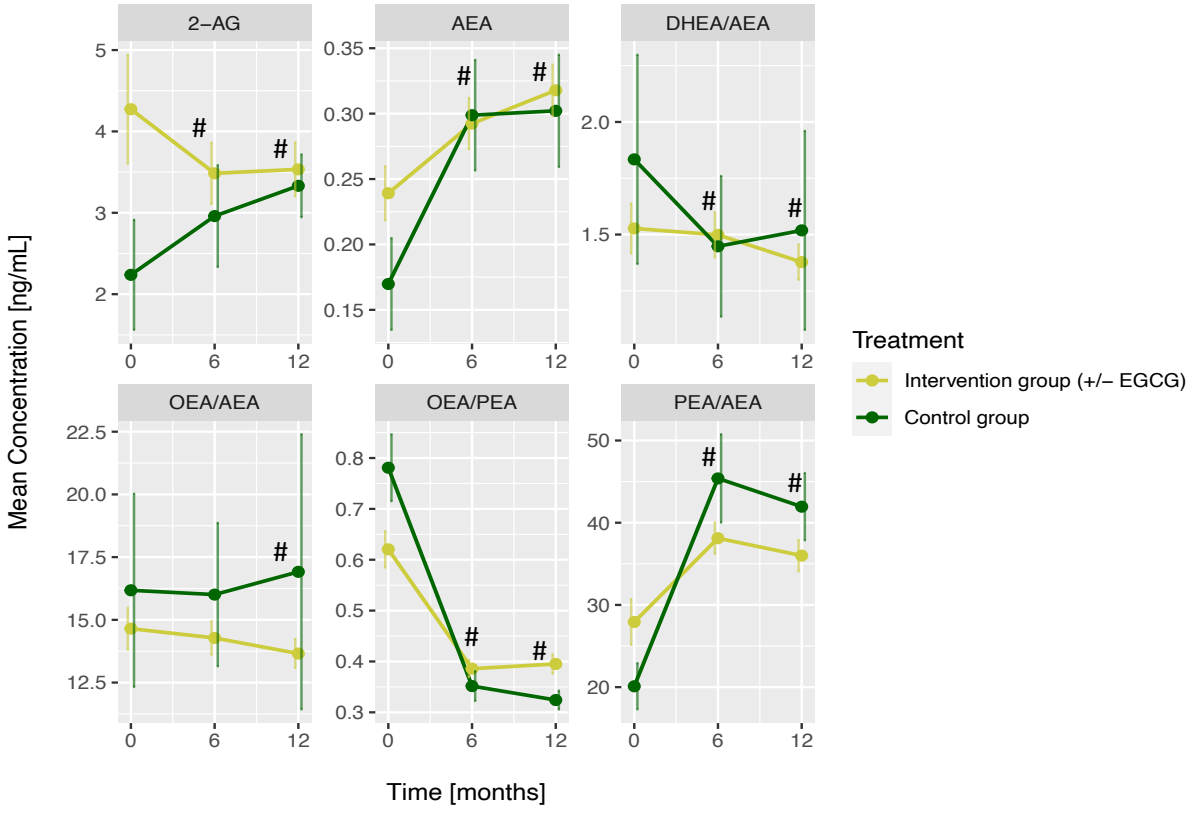


Figure 9: Effect of multimodal treatment
Plot displays the mean plasmatic endocannabinoid concentrations [ng/mL] at each time stamp (0, 6 and 12 months). Plot is grouped by A+B vs. the control group C. Error bars display standard deviation and a confidence interval of 95 % was chosen.
Statistical significance after 6 and 12 months compared to baseline was tested with a linear mixed-effects model ($p<0.05$). Statistically significant results are displayed with (#).

The modulation of EC concentrations during the intervention is showed in **figure 9**. Thus, comparing overall EC and specific EC ratio concentrations, the following trend was detected: PEA/AEA > OEA/AEA > 2-AG > AEA. Regarding temporal development of EC concentrations,

the same conclusion as comparing sex differences could be observed; different compounds show individual tendencies. 2-AG shows an unusual gap in baseline EC concentration, which cannot be seen in the other compounds/ratios. Accordingly, it's not possible to evaluate if 2-AG experiences an increase or decrease. For AEA, DHEA/AEA and OEA/PEA an ascending trend was determined, whereas OEA/PEA and PEA/AEA show a descending trend. In the case of OEA/PEA, the compared groups (A+B vs. C) behave almost parallel between 0 and 6 months before moving in opposite directions. In contrast to the control group, the intervention groups increase in concentrations.

All compounds/ratios display a significant difference between treatment arm and control group when comparing 0-6 months and 0-12 months, except for OEA/AEA between baseline and 6 months (p=0.8595) . P values are given in the table below **(Table 5)**.

Table 6: P-values of longitudinal EC concentrations divided by treatment A+B vs. C

Compound	*2-AG*	*AEA*	*DHEA/AEA*	*OEA/AEA*	*PEA/AEA*	*OEA/PEA*
P-value 0-6	0.0169	0.0063	0.0112	0.8595	4.1574E-05	4.9064E-05
P-value 0-12	0.0087	0.0467	0.0239	0.0416	0.0003	2.3775E-06

3.4.2 Longitudinal analysis of differences between treatment groups A and B

As previously mentioned, clinical trial and thus information on allocation of EGCG or placebo supplementation to the groups A and B is still masked. The question whether there is a difference between them can still be investigated. It was found out, that study arms A and B were statistically significant between 0 and 6 months as well as between 0 and 12 months for all compounds/ratios except for OEA/AEA **(Figure 10, Table 6)**.

This indicates that EGCG could possibly alter the endocannabinoid concentrations. However, at this time no statement on which of the treatment groups is supplemented with EGCG can be made.

Table 7: P-values of longitudinal EC concentrations divided by treatment A vs. B

Compound	*2-AG*	*AEA*	*DHEA/AEA*	*OEA/AEA*	*PEA/AEA*	*OEA/PEA*
P-value 0-6	0.0012	0.0026	0.0357	0.2861	0.0035	0.0002
P-value 0-12	0.0012	0.0027	0.0161	0.3394	0.0029	0.0003

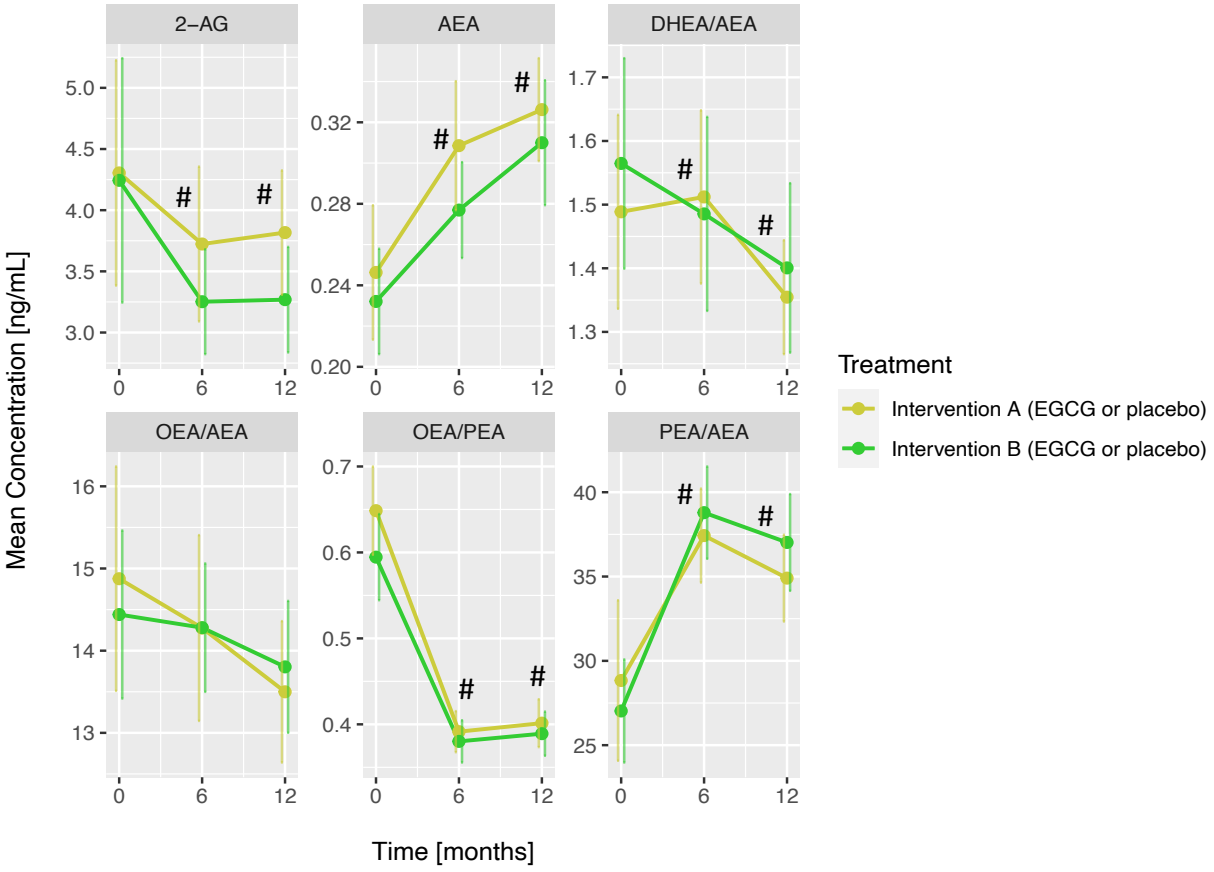


Figure 10: Difference among multimodal intervention groups
Plot displays the mean plasmatic endocannabinoid concentrations [ng/mL] at each time stamp (0, 6 and 12 months) aligning the two treatment groups (EGCG or placebo). Error bars display standard deviation and a confidence interval of 95 % was chosen.
Statistical significance after 6 and 12 months compared to baseline was tested with a linear mixed-effects model ($p<0.05$). Statistically significant results are displayed with (#).

3.5 Influence of multimodal intervention on cardiovascular risk

As shown in **Figure 11+12,** the mean course of the TyG index develops in a decreasing manner. When analysing change over time between females and males, significant difference between 0 and 12 months was found (p=0.0467). Testing if treatment groups differ, only a marginal difference was detected (p=0.0761) **(Table 7)**.

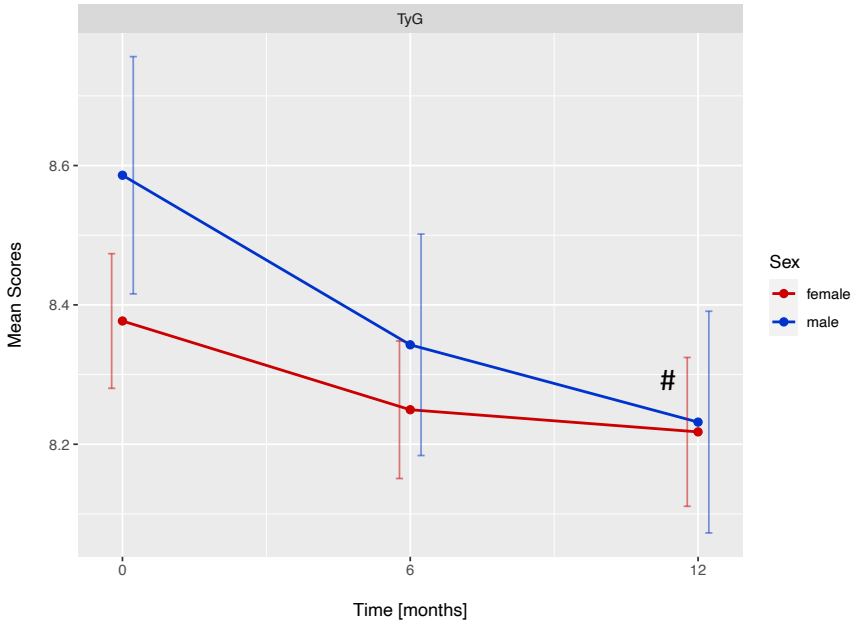


Figure 11: Change in TyG score over time divided by sex
Plot displays the mean TyG scores at each time stamp (0, 6 and 12 months). Plot is grouped by A+B vs. the control group C. Error bars display standard deviation and a confidence interval of 95 % was chosen.
Statistical significance after 6 and 12 months compared to baseline was tested with a linear mixed-effects model ($p<0.05$). Statistically significant results are displayed with (#).

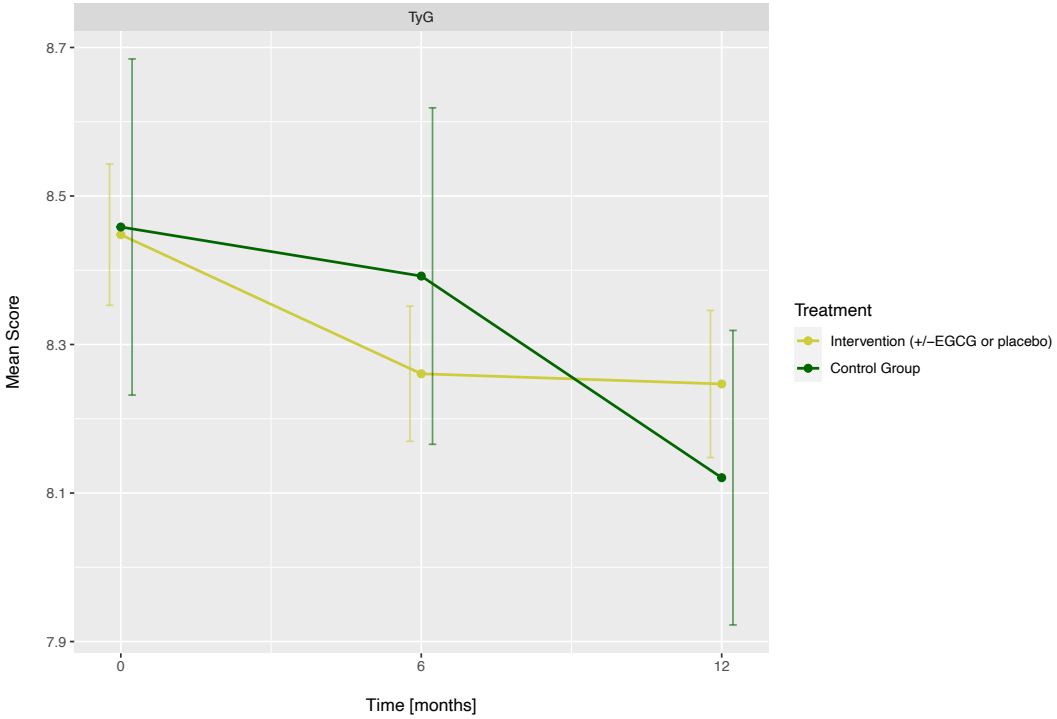


Figure 12: Change in TyG score over time divided by treatment
Plot displays the mean TyG scores at each time stamp (0, 6 and 12 months). Plot is grouped by A+B vs. the control group C. Error bars display standard deviation and a confidence interval of 95 % was chosen.
Statistical significance after 6 and 12 months compared to baseline was tested with a linear mixed-effects model ($p<0.05$). Statistically significant results would have been displayed with (#).

Table 8: P-values of longitudinal TyG analysis grouped by sex and treatment

	Grouped by sex	*Grouped by treatment*
P-value 0-6	0.0760	0.2408
P-value 0-12	0.0467	0.0761

3.6 Longitudinal cognitive analysis

Analysis of cognitive performance of the study participants consisted of three different assessment tests, each grouped by sex and treatment arm. As before, illustrations of sex do not include participants administered to the control group and respectively graphs of treatment evaluation display A+B in comparison to the controls.

3.6.1 ADCS-PACC and ADCS-PACC-Plus-exe

ADCS-PACC and ADCS-PACC-Plus screening divided by sex and treatment seem to have an increasing tendency **(Supplementary figure 1-4)**. Nevertheless, statistical testing in both cases did not prove to be not significant **(Supplementary table 1+2)**.

3.6.2 MOCA

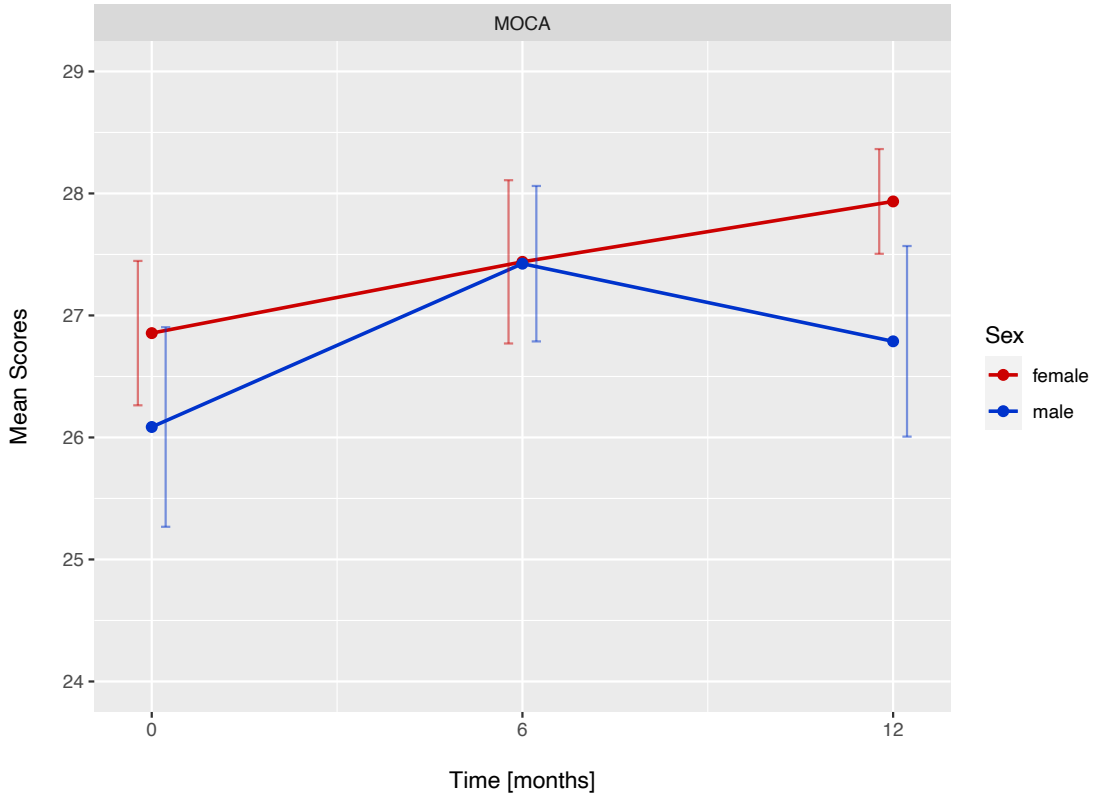


Figure 13: Change in MOCA score over time divided by sex
Plot displays the mean MOCA score at each time stamp (0, 6 and 12 months) with exclusion of participants administered to the control group. Error bars display standard deviation and a confidence interval of 95 % was chosen.
Statistical significance after 6 and 12 months compared to baseline was tested with a linear mixed-effects model ($p<0.05$). Significant results would have been displayed with (#).

Next the evaluation of cognitive performance of the study participants cognition analysis with MOCA was performed **(Figure 13+14)**. Cognition seems to be improving among time in the participants that followed the multimodal intervention, but change is not statistically significant between sex **(Table 8)**.

Table 9: P-values of longitudinal MOCA analysis grouped by sex and treatment

	Grouped by sex	***Grouped by treatment***
P-value 0-6	0.3053	0.0321
P-value 0-12	0.4218	0.0523

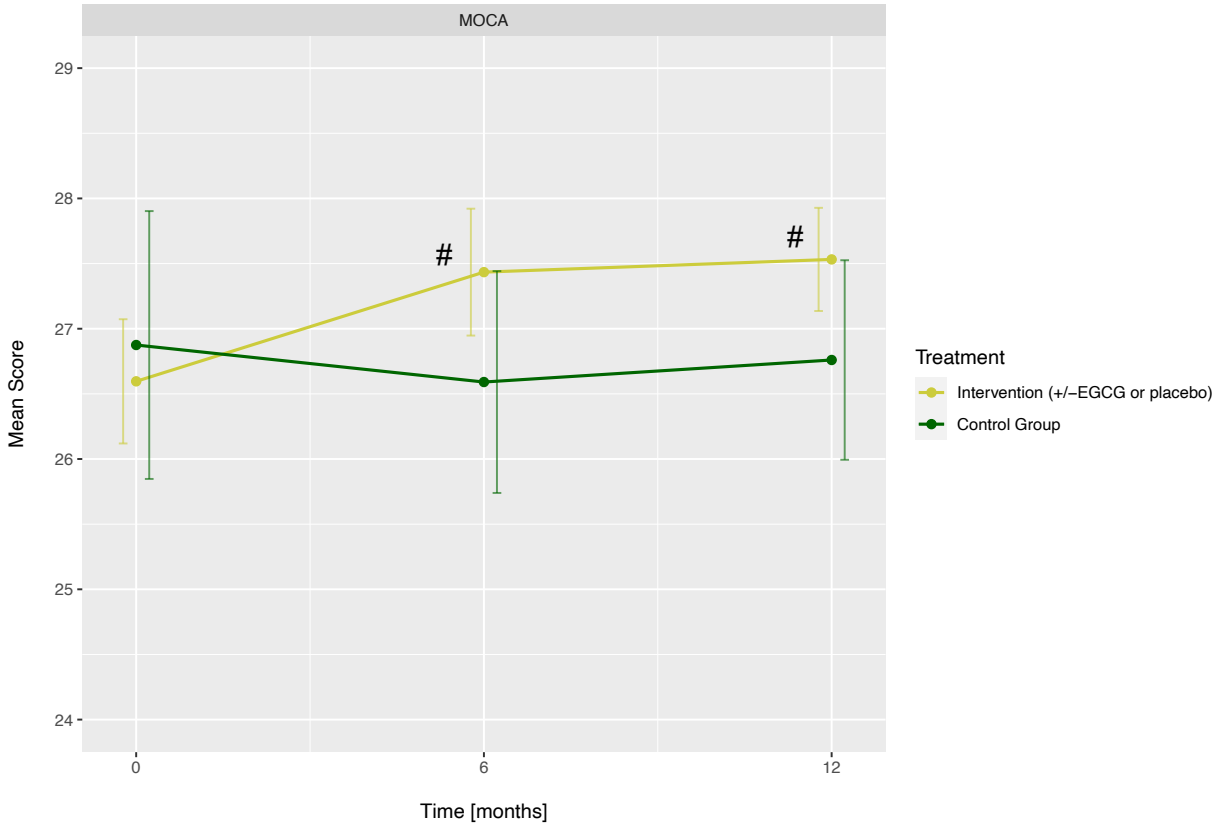


Figure 14: Change in MOCA score over time divided by treatment
Plot displays the mean MOCA score at each time stamp (0, 6 and 12 months). Plot is grouped by A+B vs. the control group C. Error bars display standard deviation and a confidence interval of 95 % was chosen. Statistical significance after 6 and 12 months compared to baseline was tested with a linear mixed-effects model ($p<0.05$). Statistically significant results are displayed with (#).

Analysis of treatment influence on cognition measured with MOCA, a statistical significance after 6 ($p=0.0321$) and 12 months ($p=0.0523$) in comparison to baseline has been detected **(Table 8).**

4 DISCUSSION

The ECS is a complex system, involved in many regulatory functions and research is implying a connection between deregulation of the ECS and the development of neurodegenerative diseases such as AD (Bisogno et al., 2021). The present study aimed to gather a deeper understanding of these alterations. The corresponding hypothesis of this thesis was, that over time the implemented multimodal intervention of the PENSA study, can impact the distributed amounts of EC and EC related ratios, consequently improve cognition and thus lower the risk for AD.

The findings on differences in circulating concentrations of target ECs over 12-months between interventions (+/-EGCG) compared to individuals only following lifestyle recommendations (control group) indicate that, multimodal interventions (PENSA-study) have a statistically significant influence on the plasmatic levels of all investigated ECs and EC ratios **(Table 5)**. Suggesting that a MedDiet, physical activity, cognitive training and specific social training can modulate the ECS **(Figure 9)**. Decrease of 2-AG and increase of AEA are in accordance to results from other clinical trials focusing on lifestyle interventions (Soldevila-Domenech et al., 2022). Furthermore, data on distribution of 2-AG and AEA, considering overall concentrations correspond with previous findings, stating that 2-AG has a higher plasma abundance (Zou & Kumar, 2018).
Longitudinal analysis also showed unusual baseline (0 months) EC concentration of 2-AG in view of compared groups **(Figure 10)**. Even though study individuals are not expected to differ at the start of the trial, EC concentrations e.g., are strongly impacted by weight. A possibility could be that individuals of the control group display higher BMI scores. However, further inspection of this issue is requested.

Investigation of variations among interventions (+/-EGCG) to obtain information on effectiveness of EGCG, have delivered statistically significant differences in comparison over 12-months **(Figure 10+Table 6)**. Nonetheless, these results cannot be credited to the assumed positive effect of EGCG, even though (Youn et al., 2022) have pointed out the neuroprotective properties of EGCG regarding AD. This correlation requires disclosure of unblinding the study after completion of the trial and further research.

Participants following the multimodal intervention also showed a decrease of cardiovascular risk evaluated with the TyG index compared to controls. Nevertheless, findings are not statistically significant. For future work, it could be hypothesised if a longer study design could lead to more informative results.

Cognitive performance was evaluated through ADCS-PACC, ADCS-PACC-Plus-exe and MOCA and higher scores indicate better cognition. In all cases, increasing tendencies where found **(Figure 13+14, Supplementary figure 1-4),** pointing towards improvement of cognition but a statistical significance **(Table 8)** was only found preliminarily in MOCA following treatment groups after 6 and 12 months in comparison to baseline cognition. It is also important, that PENSA participants are healthy individuals and although cognition improved, it was limited to a maximum of one point and no correlation with EC concentrations or the TyG index was proven.

Analysing sex differences in view of, baseline EC concentrations, longitudinal modification of EC concentrations as well as TyG scores and cognition scores, resulted in the overall statement, that there is no relevant difference in our case, contradictory to other studies (Soldevila-Domenech et al., 2022). While comparing the results to previous research that involve interventions, it must be pointed out that a high heterogeneity in EC concentration development tendencies exists. This might be explained by the wide range of interventions (e.g. intensity or type of chosen physical activity) or the population chosen for each study (APOE genotype; overweight/obese or healthy BMI), which make it difficult to directly compare results. Longitudinal progression of cognition and TyG scores manifested in an expected manner but due to non-low existing statistical significance multimodal intervention design could be altered in view of duration for future studies (Charytoniuk et al., 2022).

Due to the fact, that the multimodal intervention design of the PENSA study had an impact on the endocannabinoid system, the main hypothesis proves to be correct. Modulation of EC concentrations neither correlated with improvement of cognition nor with the decrease of cardiovascular risk, thus these findings cannot be attributed to the ECS alone. It is important to have in mind that PENSA study participants are a healthy population with neither non-pathological cardiovascular risk nor cognitive score indicating an impaired cognition. Thus, although the multimodal intervention has proven to be effective to reduce cardiovascular risk

and improve cognitive performance of the participants, interpretation of statistical correlations may be difficult, since participants are within normal parameters.

Nonetheless the increasing number of studies linking the ECS to neurological diseases suggests, that due to the complexity of Alzheimer`s disease and the ECS itself, further investigations are needed to fully understand the underlying mechanisms and the possible connection between them. Given the absence of any effective treatment addressing the pathological changes associated with AD, it becomes even more crucial to emphasize the significance of investigating in preventive strategies to successfully combat the disease.

5 CONCLUSION

To conclude the multimodal intervention implemented in the PENSA study, significantly altered the evaluated plasmatic endocannabinoid concentrations of participants with SCD and the ε4 variant of the APOE gene, with differences found among treatment arms (+/- EGCG). Cardiovascular risk was decreased while cognitive performance was ameliorated and regarding sex differences no consequential results could be found. To further develop and confirm the presented findings, future research on investigating the connection between AD, the ECS and cardiovascular risk and how they can be influenced by specifically designed interventions should focus on establishing guidelines to facilitate the comparison of results.

6 REFRENCES

Andrade, C. (2021). Z Scores, Standard Scores, and Composite Test Scores Explained. *Indian Journal of Psychological Medicine*, *43*(6), 555–557. https://doi.org/10.1177/02537176211046525

Armeli, F., Bonucci, A., Maggi, E., Pinto, A., & Businaro, R. (2021). Mediterranean diet and neurodegenerative diseases: The neglected role of nutrition in the modulation of the endocannabinoid system. In *Biomolecules* (Vol. 11, Issue 6). MDPI AG. https://doi.org/10.3390/biom11060790

Armstrong, R. A. (2013). What causes alzheimer's disease? *Folia Neuropathologica*, *51*(3), 169–188. https://doi.org/10.5114/FN.2013.37702

Armstrong, R. A. (2019). Risk factors for Alzheimer's disease. In *Folia Neuropathologica* (Vol. 57, Issue 2, pp. 87–105). Termedia Publishing House Ltd. https://doi.org/10.5114/fn.2019.85929

Berry, A. J., Zubko, O., Reeves, S. J., & Howard, R. J. (2020). Endocannabinoid system alterations in Alzheimer's disease: A systematic review of human studies. *Brain Research*, *1749*. https://doi.org/10.1016/j.brainres.2020.147135

Bisogno, T., Lauritano, A., & Piscitelli, F. (2021). The endocannabinoid system: A bridge between alzheimer's disease and gut microbiota. In *Life* (Vol. 11, Issue 9). MDPI. https://doi.org/10.3390/life11090934

Breteler, M. M. B., Claus, J. J., Grobbee, D. E., & Hofman, A. (1994). *Cardiovascular disease and distribution of cognitive function in elderly people: the Rotterdam study*.

Caron, R. M., Chandrakant Gawde, N., Denetiu, I., Budreviciute, A., Damiati, S., Khdr Sabir, D., Onder, K., Schuller-Goetzburg, P., Plakys, G., Katileviciute, A., Khoja, S., & Kodzius, R. (2020). Management and Prevention Strategies for Non-communicable Diseases (NCDs) and Their Risk Factors. *Frontiers in Public Health | Www.Frontiersin.Org*, *8*, 574111. https://doi.org/10.3389/fpubh.2020.574111

Charytoniuk, T., Zywno, H., Berk, K., Bzdega, W., Kolakowski, A., Chabowski, A., & Konstantynowicz-Nowicka, K. (2022). The Endocannabinoid System and Physical Activity—A Robust Duo in the Novel Therapeutic Approach against Metabolic Disorders. *International Journal of Molecular Sciences*, *23*(6). https://doi.org/10.3390/ijms23063083

Charytoniuk, T., Zywno, H., Konstantynowicz-Nowicka, K., Berk, K., Bzdega, W., & Chabowski, A. (2020). Can physical activity support the endocannabinoid system in the preventive and therapeutic approach to neurological disorders? In *International Journal*

of Molecular Sciences (Vol. 21, Issue 12, pp. 1–16). MDPI AG. https://doi.org/10.3390/ijms21124221

Chávez, A. E., Chiu, C. Q., Castillo, P. E., & Purpura, D. P. (n.d.). *TRPV1 activation by endogenous anandamide triggers postsynaptic LTD in dentate gyrus*. https://doi.org/10.1038/nn.2684

de la Torre Fornell, R., Luis Molinuevo, J., & Forcano Gamazo, L. (2020). *Prevention of cognitive decline in APOE-ε4 carriers with Subjective Cognitive Decline after EGCG and a multimodal intervention (PENSA) Internal Reference: PENSA Protocol version: version 4 Protocol*.

De Petrocellis, L., Cascio, M. G., & Di Marzo, V. (2004). The endocannabinoid system: A general view and latest additions. In *British Journal of Pharmacology* (Vol. 141, Issue 5, pp. 765–774). https://doi.org/10.1038/sj.bjp.0705666

Donohue, M. C., Sperling, R. A., Salmon, D. P., Rentz, D. M., Raman, R., Thomas, R. G., Weiner, M., & Aisen, P. S. (2014). The Preclinical Alzheimer Cognitive Composite. *JAMA Neurology*, *71*(8), 961–970. https://doi.org/10.1001/jamaneurol.2014.803

Duong, S., Patel, T., & Chang, F. (2017). Dementia: What pharmacists need to know. *Canadian Pharmacists Journal*, *150*(2), 118–129. https://doi.org/10.1177/1715163517690745

Elliott, R. (n.d.). *Executive functions and their disorders*. https://doi.org/10.1093/bmb/ldg65.049

Fareed, M., & Chaudhary, A. A. (2022). Herbal medicines for diabetes: Insights and recent advancement. *Herbal Medicines: A Boon for Healthy Human Life*, 207–222. https://doi.org/10.1016/B978-0-323-90572-5.00026-3

Forcano, L., Fauria, K., Soldevila-Domenech, N., Minguillón, C., Lorenzo, T., Cuenca-Royo, A., Menezes-Cabral, S., Pizarro, N., Boronat, A., Molinuevo, J. L., & Torre, R. de la. (2021). Prevention of cognitive decline in subjective cognitive decline APOE ε4 carriers after EGCG and a multimodal intervention (PENSA): Study design. *Alzheimer's and Dementia: Translational Research and Clinical Interventions*, *7*(1). https://doi.org/10.1002/trc2.12155

Fratiglioni, L., Winblad, B., & von Strauss, E. (2007). Prevention of Alzheimer's disease and dementia. Major findings from the Kungsholmen Project. *Physiology and Behavior*, *92*(1–2), 98–104. https://doi.org/10.1016/j.physbeh.2007.05.059

Gallego-Landin, I., García-Baos, A., Castro-Zavala, A., & Valverde, O. (2021). Reviewing the Role of the Endocannabinoid System in the Pathophysiology of Depression. In *Frontiers*

in Pharmacology (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fphar.2021.762738

Gouras, G. K., Olsson, T. T., & Hansson, O. (2014). *β-amyloid Peptides and Amyloid Plaques in Alzheimer's Disease*. https://doi.org/10.1007/s13311-014-0313-y

Hippius, H., & Neundörfer, G. (2003). The discovery of Alzheimer's disease. *Dialogues in Clinical Neuroscience*, *5*(1), 101–108. https://doi.org/10.31887/dcns.2003.5.1/hhippius

Jessen, F., Amariglio, R. E., Buckley, R. F., van der Flier, W. M., Han, Y., Molinuevo, J. L., Rabin, L., Rentz, D. M., Rodriguez-Gomez, O., Saykin, A. J., Sikkes, S. A. M., Smart, C. M., Wolfsgruber, S., & Wagner, M. (2020). The characterisation of subjective cognitive decline. In *The Lancet Neurology* (Vol. 19, Issue 3, pp. 271–278). Lancet Publishing Group. https://doi.org/10.1016/S1474-4422(19)30368-0

Jessen, F., Amariglio, R. E., Van Boxtel, M., Breteler, M., Ceccaldi, M., Chételat, G., Dubois, B., Dufouil, C., Ellis, K. A., Van Der Flier, W. M., Glodzik, L., Van Harten, A. C., De Leon, M. J., McHugh, P., Mielke, M. M., Molinuevo, J. L., Mosconi, L., Osorio, R. S., Perrotin, A., … Wagner, M. (2014). A conceptual framework for research on subjective cognitive decline in preclinical Alzheimer's disease. *Alzheimer's and Dementia*, *10*(6), 844–852. https://doi.org/10.1016/j.jalz.2014.01.001

Jin, J.-L., Cao, Y.-X., Wu, L.-G., You, X.-D., Guo, Y.-L., Wu, N.-Q., Zhu, C.-G., Gao, Y., Dong, Q.-T., Zhang, H.-W., Sun, D., Liu, G., Dong, Q., & Li, J.-J. (2018). Triglyceride glucose index for predicting cardiovascular outcomes in patients with coronary artery disease. *J Thorac Dis*, *10*(11), 6137–6146. https://doi.org/10.21037/jtd.2018.10.79

Kivipelto, M., Solomon, A., Ahtiluoto, S., Ngandu, T., Lehtisalo, J., Antikainen, R., Bäckman, L., Hänninen, T., Jula, A., Laatikainen, T., Lindström, J., Mangialasche, F., Nissinen, A., Paajanen, T., Pajala, S., Peltonen, M., Rauramaa, R., Stigsdotter-Neely, A., Strandberg, T., … Soininen, H. (2013). The Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability (FINGER): Study design and progress. *Alzheimer's and Dementia*, *9*(6), 657–665. https://doi.org/10.1016/j.jalz.2012.09.012

Kleberg, K., Hassing, H. A., & Hansen, H. S. (2014). Classical endocannabinoid-like compounds and their regulation by nutrients. *BioFactors*, *40*(4), 363–372. https://doi.org/10.1002/BIOF.1158

Kowiański, P., Lietzau, G., Czuba, E., Waśkow, M., Steliga, A., & Moryś, J. (2018). BDNF: A Key Factor with Multipotent Impact on Brain Signaling and Synaptic Plasticity. *Cellular and Molecular Neurobiology*, *38*(3), 579. https://doi.org/10.1007/S10571-017-0510-4

Kristiansen, K. (2004). Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. *Pharmacology & Therapeutics*, *103*(1), 21–80. https://doi.org/10.1016/J.PHARMTHERA.2004.05.002

Lăcătușu, C. M., Grigorescu, E. D., Floria, M., Onofriescu, A., & Mihai, B. M. (2019). The mediterranean diet: From an environment-driven food culture to an emerging medical prescription. In *International Journal of Environmental Research and Public Health* (Vol. 16, Issue 6). MDPI AG. https://doi.org/10.3390/ijerph16060942

Lloret, A., Monllor, P., Esteve, D., Cervera-Ferri, A., & Lloret, M. A. (2019). Obesity as a Risk Factor for Alzheimer's Disease: Implication of Leptin and Glutamate. *Frontiers in Neuroscience*, *13*(MAY). https://doi.org/10.3389/FNINS.2019.00508

Mielke, M. M. (2018). *Sex and Gender Differences in Alzheimer's Disease Dementia*.

Namba, Y., Tomonaga, M., Kawasaki, H., Otomo, E., & Ikeda, K. (1991). Apolipoprotein E immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in Alzheimer's disease and kuru plaque amyloid in Creutzfeldt-Jakob disease. *Brain Research*, *541*(1), 163–166. https://doi.org/10.1016/0006-8993(91)91092-F

Nasreddine, Z. S., Phillips, N. A., Bédirian, V., Charbonneau, S., Whitehead, V., Collin, I., Cummings, J. L., & Chertkow, H. (n.d.). *The Montreal Cognitive Assessment, MoCA: A Brief Screening Tool For Mild Cognitive Impairment*. https://doi.org/10.1111/j.1532-5415.2005.53221.x

Pastor, A., Farré, M., Fitó, M., Fernandez-Aranda, F., De La Torre, R., De La, R., & Fornell, T. (2014). *Critical issues in the analysis of endocannabinoids and related compounds in plasma: artifactual isomerization and ex-vivo enzymatic generation of 2-monoacylglycerols*. www.jlr.org

Pazos, M. R., Núñez, E., Benito, C., Tolón, R. M., & Romero, J. (2004). Role of the endocannabinoid system in Alzheimer's disease: New perspectives. In *Life Sciences* (Vol. 75, Issue 16, pp. 1907–1915). Elsevier Inc. https://doi.org/10.1016/j.lfs.2004.03.026

Raber, J., Huang, Y., & Ashford, J. W. (2004). ApoE genotype accounts for the vast majority of AD risk and AD pathology. *Neurobiology of Aging*, *25*(5), 641–650. https://doi.org/10.1016/J.NEUROBIOLAGING.2003.12.023

Rodríguez Fonseca, F. DE, Del Arco, I., Javier Bermudez-silva, F., Bilbao, A., Cippitelli, A., & Navarro, M. (2005). THE ENDOCANNABINOID SYSTEM: PHYSIOLOGY AND

PHARMACOLOGY. *Alcohol & Alcoholism*, *40*(1), 2–14. https://doi.org/10.1093/alcalc/agh110

Rosenberg, A., Mangialasche, F., Ngandu, T., Solomon, A., & Kivipelto, M. (2020). Brief reports Multidomain Interventions to Prevent Cognitive Impairment, Alzheimer's Disease, and Dementia: From FINGER to World-Wide FINGERS. *The Journal of Prevention of Alzheimer's Disease-JPAD*, *7*(1), 29–36. https://doi.org/10.14283/jpad.2019.41

Serrano-Pozo, A., Frosch, M. P., Masliah, E., & Hyman, B. T. (n.d.). *Neuropathological Alterations in Alzheimer Disease*. https://doi.org/10.1101/cshperspect.a006189

Serrano-Pozo, A., Frosch, M. P., Masliah, E., & Hyman, B. T. (2011). Neuropathological alterations in Alzheimer disease. *Cold Spring Harbor Perspectives in Medicine*, *1*(1). https://doi.org/10.1101/cshperspect.a006189

Sharifi-Rad, J., Rapposelli, S., Sestito, S., Herrera-Bravo, J., Arancibia-Diaz, A., Salazar, L. A., Yeskaliyeva, B., Beyatli, A., Leyva-Gómez, G., González-Contreras, C., Gürer, E. S., Martorell, M., & Calina, D. (2022). Multi-Target Mechanisms of Phytochemicals in Alzheimer's Disease: Effects on Oxidative Stress, Neuroinflammation and Protein Aggregation. *Journal of Personalized Medicine*, *12*(9), 12. https://doi.org/10.3390/JPM12091515

Simon, V., & Cota, D. (2017). Mechanisms in endocrinology: Endocannabinoids and metabolism: Past, present and future. In *European Journal of Endocrinology* (Vol. 176, Issue 6, pp. R309–R324). BioScientifica Ltd. https://doi.org/10.1530/EJE-16-1044

Soldevila-Domenech, N., Pastor, A., Sala-Vila, A., Lázaro, I., Boronat, A., Muñoz, D., Castañer, O., Fagundo, B., Corella, D., Fernández-Aranda, F., Martínez-González, M. Á., Salas-Salvadó, J., Fitó, M., & de la Torre, R. (2022). Sex differences in endocannabinoids during 3 years of Mediterranean diet intervention: Association with insulin resistance and weight loss in a population with metabolic syndrome. *Frontiers in Nutrition*, *9*. https://doi.org/10.3389/fnut.2022.1076677

Stampfer, M. J. (2006). Cardiovascular disease and Alzheimer's disease: Common links. In *Journal of Internal Medicine* (Vol. 260, Issue 3, pp. 211–223). https://doi.org/10.1111/j.1365-2796.2006.01687.x

Tantimonaco, M., Ceci, R., Sabatini, S., Catani, M. V., Rossi, A., Gasperi, V., & Maccarrone, M. (2014). Physical activity and the endocannabinoid system: an overview. *Cell. Mol. Life Sci*, *71*, 2681–2698. https://doi.org/10.1007/s00018-014-1575-6

Tao, L. C., Xu, J. ni, Wang, T. ting, Hua, F., & Li, J. J. (2022). Triglyceride-glucose index as a marker in cardiovascular diseases: landscape and limitations. *Cardiovascular Diabetology*, *21*(1), 1–17. https://doi.org/10.1186/S12933-022-01511-X/FIGURES/1

Thomann, A. E., Goettel, N., Monsch, R. J., Berres, M., Jahn, T., Steiner, L. A., & Monsch, A. U. (2018). The Montreal Cognitive Assessment: Normative Data from a German-Speaking Cohort and Comparison with International Normative Samples. *Journal of Alzheimer's Disease*, *64*(2), 643–655. https://doi.org/10.3233/JAD-180080

Vaz, M., & Silvestre, S. (2020). Alzheimer's disease: Recent treatment strategies. *European Journal of Pharmacology*, *887*, 173554. https://doi.org/10.1016/J.EJPHAR.2020.173554

von Forth, B. W., Henschler, D., & Rummel, W. (2017). *Pharmakologie und Toxikologie ELSE VIER*.

Weir, C. B., & Jan, A. (2019). BMI Classification Percentile And Cut Off Points. *StatPearls*. http://europepmc.org/books/NBK541070

WHO. (n.d.). *Cardiovascular diseases*. Retrieved May 21, 2023, from https://www.who.int/health-topics/cardiovascular-diseases#tab=tab_1

Wolfram, S. (2013). Effects of Green Tea and EGCG on Cardiovascular and Metabolic Health. *Http://Dx.Doi.Org/10.1080/07315724.2007.10719626*, *26*(4), 373S-388S. https://doi.org/10.1080/07315724.2007.10719626

Wood, L., Tai, L. M., Anusuyadevi, M., Plourde, M., Husain, M. A., & Laurent, B. (2021). *APOE and Alzheimer's Disease: From Lipid Transport to Physiopathology and Therapeutics*. https://doi.org/10.3389/fnins.2021.630502

Yoo, S., Kim, K., Nam, H., & Lee, D. (2018). *Discovering Health Benefits of Phytochemicals with Integrated Analysis of the Molecular Network, Chemical Properties and Ethnopharmacological Evidence*. https://doi.org/10.3390/nu10081042

Youn, K., Ho, C. T., & Jun, M. (2022). Multifaceted neuroprotective effects of (-)-epigallocatechin-3-gallate (EGCG) in Alzheimer's disease: an overview of pre-clinical studies focused on β-amyloid peptide. *Food Science and Human Wellness*, *11*(3), 483–493. https://doi.org/10.1016/J.FSHW.2021.12.006

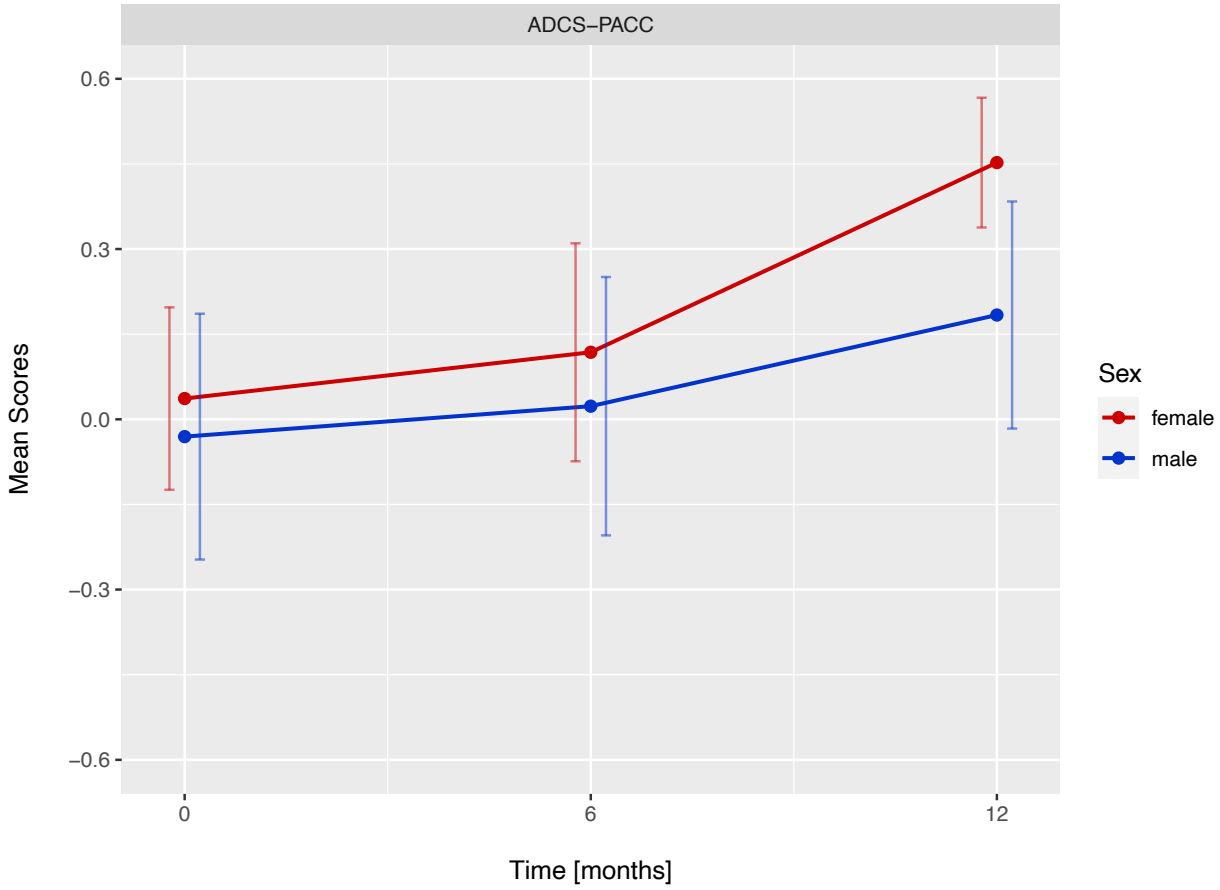
Zou, S., & Kumar, U. (2018). *Molecular Sciences Cannabinoid Receptors and the Endocannabinoid System: Signaling and Function in the Central Nervous System*. https://doi.org/10.3390/ijms19030833

7 FIGURES AND TABLES

SUPPLEMENTARY FIGURES + TABLES

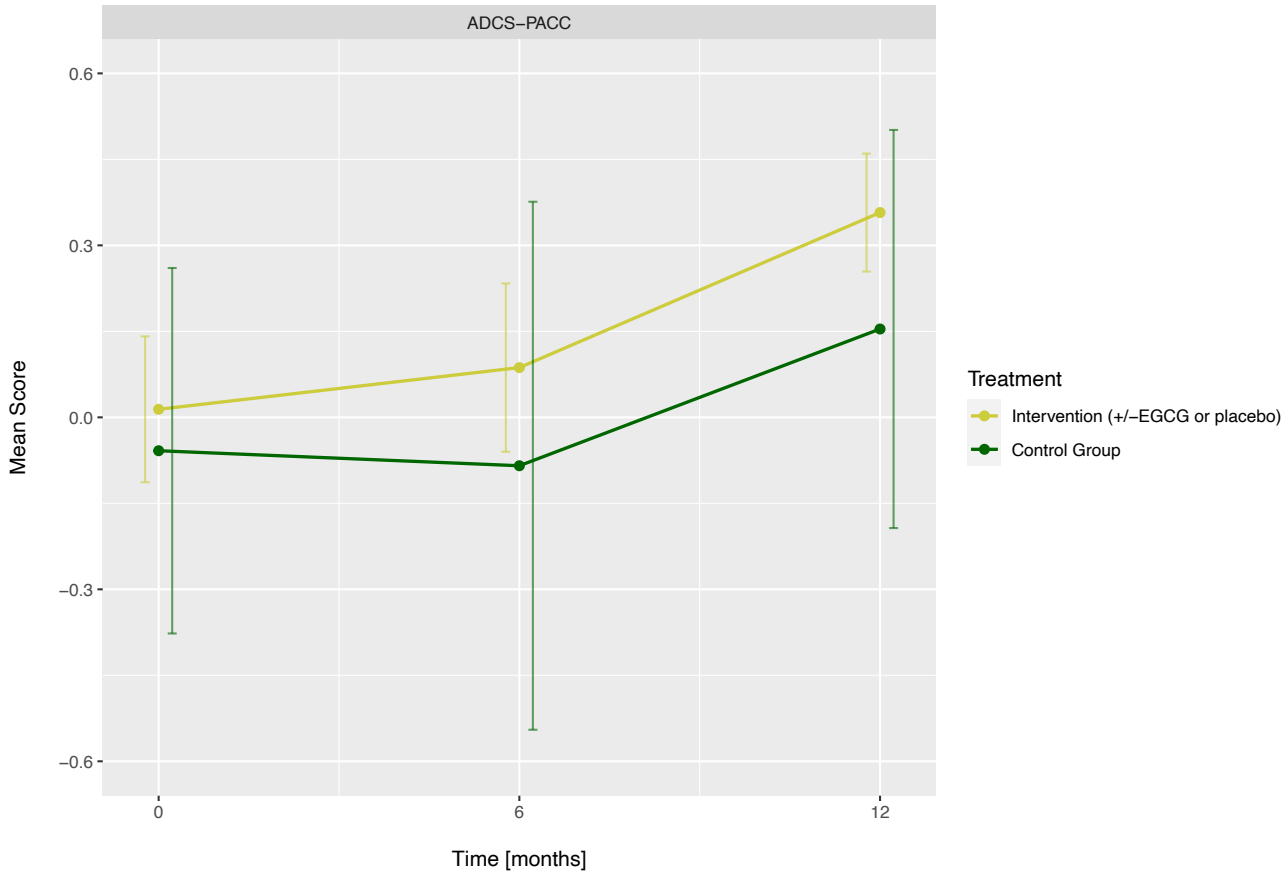
Figures

Supplementary figure 1: Cognitive screening with ADCS-PACC by sex



Supplementary figure 1: Change in ADCS-PACC score over time divided by sex
Plot displays the mean ADCS-PACC score at each time stamp (0, 6 and 12 months) with exclusion of participants administered to the control group. Error bars display standard deviation and a confidence interval of 95% was chosen.
Statistical significance after 6 and 12 months compared to baseline was tested with a linear mixed-effects model ($p<0.05$). Significant results would have been displayed with (#).

Supplementary figure 2: Cognitive screening with ADCS-PACC by treatment



Supplementary figure 2: Change in ADCS-PACC score over time divided by treatment
Plot displays the mean ADCS-PACC score at each time stamp (0, 6 and 12 months) with exclusion of participants administered to the control group. Error bars display standard deviation and a confidence interval of 95% was chosen.
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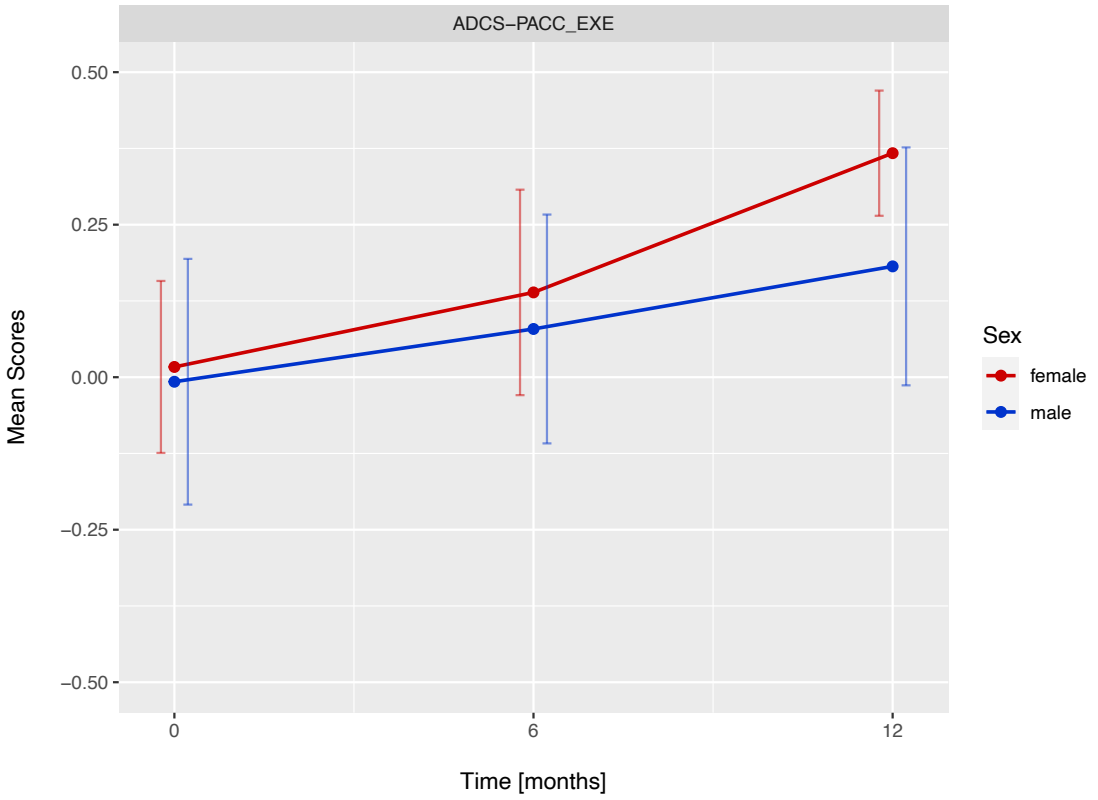
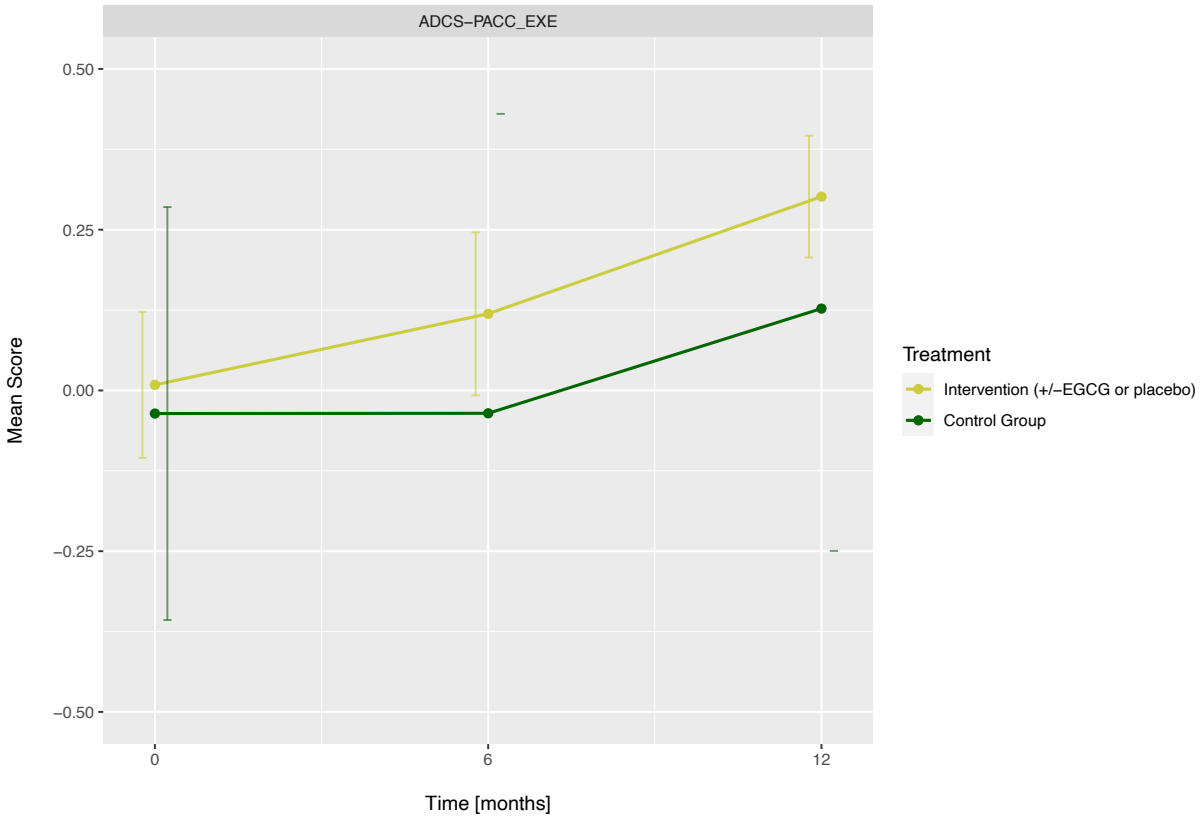
Supplementary figure 3: Cognitive screening with ADCS-PACC-Plus-exe by sex



Supplementary figure 3: Change in ADCS-PACC-exe score over time divided by sex
Plot displays the mean ADCS-PACC-exe score at each time stamp (0, 6 and 12 months) with exclusion of participants administered to the control group. Error bars display standard deviation and a confidence interval of 95% was chosen.
Statistical significance after 6 and 12 months compared to baseline was tested with a linear mixed-effects model ($p<0.05$). Significant results would have been displayed with (#).

Supplementary figure 4: Cognitive screening with ADCS-PACC-Plus-exe by treatment



Supplementary figure 4: Change in ADCS-PACC-exe score over time divided by treatment
Plot displays the mean ADCS-PACC-Plus-exe score at each time stamp (0, 6 and 12 months) with exclusion of participants administered to the control group. Error bars display standard deviation and a confidence interval of 95% was chosen.
Statistical significance after 6 and 12 months compared to baseline was tested with a linear mixed-effects model ($p<0.05$). Significant results would have been displayed with (#).

Tables

Supplementary Table 1: P-values for ADCS-PACC and ADCS-PACC-Plus exe of longitudinal analysis divided by sex

Supplementary table 1: P-Values of longitudinal ADCS-PACC and ADCS-PACC-Plus-exe analysis divided by sex

Cognitive test	*ADCS-PACC*	*ADCS-PACC-Plus-exe*
P-value 0-6	0.8269	0.8345
P-value 0-12	0.2682	0.1297

Supplementary Table 2: P-values for ADCS-PACC and ADCS-PACC-Plus exe of longitudinal analysis divided by treatment

Supplementary table 2: P-values of longitudinal ADCS-PACC and ADCS-PACC-Plus-exe analysis divided by treatment

Cognitive test	*ADCS-PACC*	*ADCS-PACC-Plus-exe*
P-value 0-6	0.3302	0.1289
P-value 0-12	0.2518	0.1608