Biomarkers and cognitive function in Down syndrome

Validation for phenotyping cognitive impairment

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TESI DOCTORAL UPF / 2016

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"Ah, not in knowledge is happiness, but in the acquisition of knowledge! In forever knowing, we are forever blessed; but to know all, were the curse of a fiend."

The Power of Words – Edgar Allan Poe

Acknowledgments

So, don't you worry, all things must end.
There are sunlight uplands around the river bend.
Glorious You-Frank Turner

Not too long ago, a friend told me that he thought that no one ever read the thesis acknowledgements, that they remained unread in plain sight. Needless to say, he was surprised when he found out it wasn't the case. Almost at the same time, talking to another friend, I joked that I would change the topic of my thesis and make it about unicorns. He dared me to. So, this thesis is about unicorns. Maybe not about unicorns, but unicorns did help with it. This thesis is for all the unicorns that have helped me endure this, for all the unicorns that have been there for me.

D'una banda m'agradaria donar les gràcies a la meva "família" acadèmica, els meus "pares" als que he vist més que als de veritat. El Rafa i la Mara. El Dr. de la Torre i la Dra. Dierssen. Moltes gràcies, Rafa, per donar-me l'oportunitat de participar en aquest projecte quan vaig venir a veure't essent encara una estudiant de carrera. Per deixar-me triar i donar-me llibertat. Per la teva confiança i tot el que has aguantat, que tots sabem que no me n'he callat ni una i que als congressos em poso insuportable. Gràcies pels consells, també, fins i tot aquell famós de: "Busca't un *novio* ric". Mara, a ti también tengo mucho que agradecerte, aceptaste ser la codirectora de esta tesis casi sin conocerme. Gracias a ti he dejado de ser la chica asustadiza que temía cuestionar nada. Como no te cansas de repetirme, he crecido y he madurado científicamente y no podría haberlo hecho sin ti. Has sido dura cuando ha sido necesario, pero también comprensiva en los momentos en los que ya no podía más, gracias por tu supervisión.

D'altra banda la meva família de debò. Els meus pares, el meu germà, els meus avis. *Papa*, he heretat moltes coses de tu, entre altres el caràcter i la tendència a parlar "malament", però també la teva tossuderia, i la teva passió per fer allò que m'agrada. Ja em diràs de quina altra manera hagués pogut arribar fins aquí, sinó. Gràcies per les estones en bicicleta, esquiant, o remant pel llac mentre jo nedava. I

pels dies en els que no em deixaves anar amb bici perquè havia d'anar de congrés i "no fos cas que et tornessis a trencar alguna cosa i el Rafa em *foti bronca*". *Mama*, gràcies per ser la paciència personificada, que amb mi te'n fa falta molta. Gràcies pels "Bon dia" de cada dia i per preocupar-te de mi quan m'he posat malalta, encara que no t'ho digués. Gràcies per les estones en que aconseguies que ningú em molestés mentre escrivia a casa. Gràcies per tot. Joan, *Hodor*, com a bons germans ens barallem sovint, però com a bons germans també ens donem suport l'un a l'altre, i no podríem viure l'un sense l'altre. Ets el meu germà preferit, i l'únic que tinc. *Iaies* Maria i Isabel, avi Jordi, tots tres heu estat donant-me suport encara que no acabéssiu d'entendre de què anava tot plegat, gràcies. Avi Lluís, sé que t'hauria agradat poder veure'm en aquest moment.

A més, aquesta tesi no hagués estat possible sense l'ajuda de molta gent amb qui he compartit despatx i laboratori. Sobretot amb la gent que ha estat implicada en l'assaig clínic.

Aida, has estat una figura de suport fonamental, no sé com m'ho hauria fet sense tu, sense les teves paraules d'ànim, sense les teves abraçades en els moments en els que ja no podia més. Sempre em quedaran a la memòria les nostres estones intentant desxifrar el perquè de tot plegat i els dies de posar-nos a cantar a primera hora del matí. Sé que em trobaràs a faltar, jo a tu també, ja em diràs on trobaré algú com tu.

Lau, como dices tú en tu tesis, esta tesis es mitad tuya. Todo lo que podría decir ya lo has escrito tú, a este nivel nos compenetramos. Con tus idees de bombero y tu energía, has sido un motor de motivación y un rayo de sol que han luchado contra mi pesimismo generalizado. No sé lo que nos depara el futuro, pero lo que sí que sé es que me gustaría seguir trabajando contigo.

Joan, ¡lo que llegamos a sufrir durante el TESDAD! Las primerísimas horas, los walkies que tenían interferencias de las ambulancias. Ha valido la pena, gracias por cargarte el estudio a las espaldas. Susana, tot i que venim de mons diferents, hem après molt l'una de l'altra. Sempre recordaré els teus consells de ser més expeditiva. Gime, el TESDAD también tiene mucha parte de ti, y no sólo porque te tocara batallar con el manuscrito, sino porque te ha tocado batallar con todo, gracias por tus esfuerzos.

Klaus, *danke*. Crec que en aquesta tesi es pot apreciar l'influencia que has tingut. Sense tu, i les hores que ens hem passat discutint mètodes d'anàlisi estadístic per a repetir-lo una vegada i una altre, no hauria arribat enlloc. Guardaré les sintaxis intercanviades per sempre, així com els mails en els que discutíem sobre la veritable utilitat del p valor o sobre la utilitat de determinades proves estadístiques.

I sobretot a les famílies i als voluntaris que van fer possible l'assaig clínic que ha format part de la meva vida durant els darrers cinc anys.

Però a l'IMIM hi ha molta més gent que m'han donat suport durant aquesta aventura. Mito, ha estat un plaer, no només has estat una de les fonts a qui sempre anava a preguntar en cas de dubte, sinó que a més hem compartit mitges maratons i aigües obertes. Neus, la bondat personificada, sense tu aquest laboratori no sé pas que faria, sempre tens un moment per tothom i les nostres batalles amb l'homocisteïna m'han ensenyat molt més del que mai hauria pensat. Cristina, lo que hemos sufrido y reído con las células, lo que hemos aprendido la una de la otra, ¡ojala te hubiera conocido antes! Ya sabes que siempre podrás preguntarme lo que sea, aunque ya no esté físicamente aquí. Maribel, *veci*, els teus consells de sobre com no tornar-me boja han estat impagables, i espero seguir contant amb ells allà on estigui (també espero que em vinguis a visitar tal com promets sempre). Cristina Llop, l'organitzadora, amb la teva paciència i constància has aconseguint que el laboratori es posi a lloc, de la mateixa manera que has aconseguit que jo posés en ordre les meves idees en moments de crisi. Jose, en tot aquest temps només ens hem posat d'acord en tres coses, però millor tres que cap, que tinguis sort. I a les noves nenes, que us estimo molt, tot i que a vegades em faci la dura. Carla, la meva "substituta", posa ordre, que no s'esvalotin, t'anirà bé, ho sé. Anna, demostres que en el pot petit hi ha bona confitura, ànims i recorda d'escriure un llibre d'anècdotes! Eulàlia, ets una de les persones més treballadores i al mateix temps més optimistes que conec, només et puc dir que endavant. Maria, tu que sempre em dius que no et preocupo, tu a mi tampoc, és complicat, però val la pena. Jordi, estàs en camí de convertir-te en un dels pilars del laboratori, promet-me que mai deixaràs de cantar amb la radio quan soni Adele.

Xess, *xurri*, ets la prova que el treball dur aporta recompenses, hem compartit poc temps, però sempre he pogut comptar amb els teus somriures. I tota la resta que m'heu patit, gràcies per la paciència.

Hi ha gent que ja no és al laboratori però que ha deixat empremta en mi. L'Ester, la Laia, la Lorena, el Pedro (¡que hubiéramos hecho sin ti!), la Samanta (la meva mestra Yoda), la Sira (que ja em va dir que tot plegat passaria), la Mariola (que sempre ho solucionava tot), i la Preeti. Δημητρουλα, Ευχαριστώ, no sólo son tus galletas las mejores del mundo, sino que además tú eres la persona más dulce que nunca he conocido. Rut, la meva primera alumna, va ser una experiència molt enriquidora poder treballar amb tu. També recordar el Jaume, que em va dir que l'avisés quan llegís la tesi, i no podrà ser, aquesta tesi té part d'ell.

Voldria estendre la meva gratitud a gent amb qui he compartit laboratori, però no grup d'investigació com la Judit, tu que has aguantat més llàgrimes que ningú, et mereixeries un monument, però, de moment, això és tot el que et puc donar. Y Gretsen, con nuestros problemas con las PCR y los geles, el estar en el laboratorio a deshoras. I a gent amb qui he compartit grup d'investigació però no laboratori, com la Georgina amb qui hem passat penes en moments puntuals i tants partits de vòlei com hem pogut. And people with whom I have shared neither research group, nor lab. We met serendipitously, Roshani, but I will have to be thankful to Tyche forever.

La parte buena de estar en dos laboratorios es que conoces al doble de gente, y esto es parte de lo que me llevo de mi tiempo en el CRG. Hemos estado luchando en esto juntas, Silvina, y saldremos de esto juntas (no como de nuestras carreras corriendo), y, aunque a veces pierda la paciencia contigo, has sido mi "enlace" en el CRG. Maria, el pilar estructural del laboratorio, tu capacidad de saber manejar cualquier situación ha sido inspiradora. Jose, tenir dos directors de tesi i les PCA ens han unit, puc dir amb total convenciment que, tot i no entendre moltes de les coses que explicaves, alguna cosa he après. Marta, Mireia, Júlia, i Linus, ànims que això ja gairebé ho teniu, us espero a l'altra banda de la línia d'arribada.

También agradecer a la Dra. Gloria Benítez-King el aceptarme en su laboratorio y su hospitalidad durante mi estancia en México. Así como al resto de personal de laboratorio por hacerme sentir como en casa aunque muchas veces no les entendiera. Especialmente a Tania, hermana mexicana, fuiste mi guía durante mi estancia y lo has seguido siendo después.

I a la gent de Risc Cardiovascular i Nutrició per la part que han tingut en aquesta tesi i per deixar-me fer servir els seus instruments. Molt especialment a la Dra. Montse Fitó i al Dani Muñoz.

Sóc una persona afortunada, ja que a part del suport dels que em veient dia a dia, he tingut una bona colla d'amics que em feien de xarxa de seguretat. Per començar, vull agrair a l'Alba i la Cris totes les hores de "teràpia" que han invertit en mi, sempre que tenia un problema estàveu allà per mi i heu hagut d'aguantar moltes tonteries (i segur que encara en queden!), sé que sempre podré comptar amb vosaltres i això val molt més que res que es pugui comprar. D'altra banda m'agradaria donar les gràcies als amics de "tota la vida" per forçar-me a sortir de casa: Edgar, Gerard, Anna, Ingrid, Mercè, i Jordi. Oleguer, of course, you have been the one who has suffered with me the most, but, from your own experience, you know it is worth fighting for what you love. També vull donar les gràcies per la part que li pertoca a la Laia, que em coneix des d'abans que cap de les dues poguéssim parlar correctament i que encara segueix aquí. I les que estaven en aquest camí amb mi quan tot just començava, les companyes de la universitat, l'Ana que ja sap que és passar per això; l'Angie i l'Anna que estan a punt d'arribar-hi; la Mònica que per a complicar-ho se'n va anar a Canadà; la Mire que és el seny; i la Míriam que és la rauxa.

Sé que pot semblar que ja no em queda ningú per a agrair, però hi ha hagut quatre persones en la meva vida que m'han influenciat per a que arribés on sóc. El primer de tots va ser el Dr. Martínez, que intentant arreglar-me em va ensenyar el valor d'intentar l'impossible. Després va venir el "Senyor" Adrià, que va ser el primer que va creure que això seria possible. També he d'agrair al Dr. Josep Eladi Baños la seva orientació durant la carrera. Y a Harry por enseñarme lo que realmente era la investigación.

Hi ha molta més gent a qui m'agradaria donar les gràcies de nom, gent que ha format part de la meva vida durant aquest temps. La gent de la coral, del gimnàs, el personal de seguretat. Tota la gent que en algun moment o altre han tingut una paraula amable o un somriure per mi.

Stories have always been an integral part of me, a contraposition of my rational part, my desire to dive into dreams. Stories have accompanied me during this journey. Pat, George, Haruki, Edgar, Howard, Jenny, Joanne, Ken, Aarto, Neil, Terry, Stephen, Isaac, and more have written the words that have transported me to other places. Places like the ones that have also played a role in shaping not only who I am, but how this thesis turned out to be. Barcelona, Montréal, and Mexico have a part of them in this thesis; they have scientifically molded me. Greece, Ireland, and the Baltic countries have given me peace and have helped me stay sane throughout. And the Sea, always the Sea. Likewise, stories have music, sometimes it is unheard, but it is always there. In this case I need to thank Tom, Yannis, Matt, Ralph, Alex, and many others for composing the score to this thesis. And Frank, for the lyrics that have meant so much, and for giving the best hugs. I can't mention stories without thanking the amazing writing community, which has built-up through the years, for their support. Specially Katie, Richard, and Carina.

As a writer, I know that all stories must come to an end. There is a point when you stop writing and the reader has to imagine how the story unfolds. Maybe there will be a sequel for the story, maybe you will never write another word about it. We have reached this point, this part of the story has finished and no one knows what lies ahead.

Með suð í eyrum við spilum endalaust

Abstract

Clinical trials seeking to improve cognitive performance are in dire need of biochemical biomarkers that reflect the processes taking place in the brain. In Down syndrome, this information is crucial to understand the progression of cognitive decline, or to evaluate the beneficial effects of a treatment, but there are few available biomarkers for this condition. We have analyzed the association between biomarkers (biochemical, genetic), executive functions, and cognitive decline, in the context of a clinical trial, taking into account extrinsic factors (education, diet) that may obscure the interpretation of the obtained results. We found that altered lipid profile or increased homocysteine concentrations in plasma are associated to worse executive functions. Additionally, increased plasma concentrations of amyloid peptides are associated to early dementia signs. I also optimized a new technique of tissue and cell culture to obtain neuronal precursors from the nasal olfactory epithelium for biomarker studies.

The results of this study should provide with new tools to evaluate treatment efficacy and cognitive decline risk in the context of clinical trials in Down syndrome.

Resum

Els assajos clínics que cerquen la millora del rendiment cognitiu tenen la necessitat de disposar de biomarcadors que reflecteixin els processos que tenen lloc en el cervell. En la síndrome de Down aquesta informació és crucial per a entendre la progressió del declini cognitiu o per a avaluar els efectes beneficiosos d'un tractament però encara hi ha pocs biomarcadors disponibles per a aquesta afectació. Hem avaluat l'associació entre biomarcadors (bioquímics, genètics), funcions executives i declini cognitiu, en el context d'un assaig clínic, tenint en compte factors extrínsecs (educació, dieta) que poden afectar la interpretació dels resultats. Els nostres demostren que un perfil lipídic alterat o unes concentracions incrementades d'homocisteïna en plasma estan associats a pitjors funcions executives. També observem una associació entre concentracions incrementades de pèptids amiloides i signes primerencs de demència. També he optimitzat una nova tècnica de cultiu tissular i cel·lular per a obtenir precursors neuronals de l'epiteli olfactiu per a l'estudi de biomarcadors.

Els resultats d'aquest estudi podrien proporcionar noves eines per a avaluar l'eficàcia de tractament i el risc de declini cognitiu en el context d'assaigs clínics en la síndrome de Down.

Foreword

The important medical and social burden of nervous system diseases contrasts with the currently limited therapeutic armamentarium and with the challenges faced when developing new therapeutic options. This situation can be explained by the conjunction of various phenomena related to the limitations of animal models, the narrow focus of research on precise pathophysiological mechanisms, and methodological issues in clinical trials. It is of uttermost importance that preclinical and clinical developments promote the use of cognitive, behavioral, biological, neurological, and neuroimaging biomarkers. In this Thesis, my aim was to identify clinically-relevant biomarkers and specific predictors of persistence and/or remission of symptoms upon treatment and to establish olfactory neuroepithelial derived precursor cells for biomarker studies. These studies were performed in the context of a Phase II trial in young adults with Down syndrome (DS), using a combined therapy of epigallocatechin-3-gallate (EGCG), and cognitive training.

Down syndrome, caused by the trisomy of chromosome 21 (1), is the main genetic cause of intellectual disability. This intellectual disability, and specially the impairment in executive functions (EF) (2, 3), leads to deficits in daily living (4). Additionally, individuals with DS have a high prevalence of Alzheimer-like neuropathology, that has a dramatic impact on cognition and their adaptive behavior (4). Of the genes present in the chromosome 21, and overexpressed in DS, we have focused on two of particular interest to explain the cognitive and neurodegenerative phenotype, the Dual-Specificity Tyrosine-(Y)-Phosphorylation Regulated Kinase 1 (*DYRK1A*) and the Amyloid

Precursor Protein (*APP*). *DYRK1A* is dosage sensitive and its overexpression is sufficient to recapitulate part of the DS phenotype in animal models, including alterations in brain morphology and learning deficits (5, 6). *APP* overexpression is considered to be the cause of Alzheimer-like disease in DS (7), as it increases the production of the amyloid monomers, a process favored by DYRK1A overexpression, known to phosphorylate and affect the metabolism of APP (8, 9).

The modulation of the excessive DYRK1A kinase activity by an inhibitor has proven to rescue the DS phenotype in primary cultures and DS mouse models (10, 11). One of them is EGCG, a polyphenol from green tea (12). This thesis has been developed in the context of a double-blind, placebo-controlled, Phase 2, single center clinical trial (TESDAD; NCT01699711) using a combination of the green-tea polyphenol EGCG and cognitive training (13). The study population included young patients with DS treated for 12 months, with 6 months of follow-up.

The TESDAD study included both neuropsychological evaluations and biomarker assessments (genetic, biochemical, and neuroimaging/ neurophysiology). Thus, it was the perfect environment to assess the usefulness of genetic and biochemical biomarkers in the evaluation of treatment efficacy. I have focused on the evaluation of the relation between cognitive tasks, functional measures, and biomarkers that 1) were modified by our treatment and could be considered efficacy biomarkers, 2) have been associated with cognition in populations without intellectual disability (ID), 3) are altered in population with DS, or 4) could modulate treatment response (genetic

polymorphisms). Specifically, we focused on biomarkers that could be predictive of the conversion to dementia in the DS population, and are sensitive to the cognitive enhancing treatment. This involved an analysis of the baseline associations between the lipid profile, homocysteine, and amyloid concentrations with different cognition and cognitive decline measures. Both lipid profile and amyloid concentrations have been previously associated with cognition, are considered to be a risk factor for dementia in the general population, and are altered in DS. In the case of beta-amyloid peptides concentrations, this alteration is due to a life-long overexpression of APP in individuals with DS, but the concrete relationship of peripheral biomarkers with dementia signs has to be defined. Homocysteine concentrations are known to be reduced in population with DS, and several studies in general population have associated increased concentrations of homocysteine to alterations in executive functions and AD.

Finally, the utility of olfactory neural precursors was explored as a novel source of surrogate biomarkers for intellectual disability with the objective to avoid two main limitations. First, blood-based biomarkers present a considerable challenge as blood is a complex tissue and it lacks direct contact with brain. Second, obtaining brain tissue from living donors is not feasible. These drawbacks can be curtailed by obtaining of cells of neuronal lineage outside the brain. One of the sources of such cells is the olfactory neuroepithelium. These cells should serve as efficacy biomarkers and should also provide new predictive biomarkers.

In summary, this thesis was aimed at 1) exploring the applicability of biomarkers of cognition that are used in the general population. In this category we include biomarkers that are also related to dementia risk. Specifically we examined if there was an association between lipid profile, cognition, and dementia risk, in our population similarly to that found in euploid adults. For this specific biomarker, due to EGCG having proven to modify lipid concentrations, we also analyzed the effect that the treatment could be having on such association. Furthermore, we studied the usefulness of peripheral biomarkers of amyloidosis as cognitive markers of dementia. Finally, we investigated the association between executive functions and homocysteine concentrations. Such association has been observed in euploid population where hyperhomocysteinemia is correlated to worse cognitive function. 2) Seeking new sources of biomarkers, in particular at setting up a new method used to obtain neuro-olfactory precursors and exploring their potential for biomarker studies.

Due to the multidisciplinary approach of this Thesis it was developed in the context of a collaboration between the Integrative Pharmacology and Systems Neuroscience group in IMIM under Dr. Rafael de la Torre supervision, and the Cellular and Systems Neurobiology group at the CRG headed by Dr. Mara Dierssen. The group led by Dr. de la Torre is specialized in human neuropharmacology, in particular in clinical trials related to this discipline, allowing the development of the part of this thesis related to peripheral biomarkers in the context of a clinical trial. On the other hand, the group directed by Dr. Dierssen is widely acknowledged for its contribution on the neurobiology of DS, both at the cellular and in

the *in vivo* levels, providing, thus the expertise to develop the method to culture neuro-olfactory precursors. Additionally, in order to learn the protocol for obtaining and culture neuro-olfactory precursors I did a three-month stay at the laboratory of Dr. Gloria Benítez-King at the National Institute of Psychiatry Ramón de la Fuente Muñiz in Mexico City, Mexico.

The results obtained in this Thesis shed light on the specific associations between cognition and peripheral biomarkers that will allow ascertaining the risk of cognitive declines or dementia.

List of Abbreviations

Aβ: amyloid-β peptide

ABAS-II: Adaptive Behavior Assessment System Second Edition

AD: Alzheimer disease

ANCOVA: Analysis of Covariance

ApoE: Apolipoprotein E

APP: Amyloid Precursor Protein

CBS: Cystathionine- β-synthase

CSF: cerebrospinal fluid

COMT: Catechol-O-methyltransferase

DAPI: 4',6-diamidino-2-phenylindole

DIV: Day *in vitro*

DMEM/F-12: Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12

DMR: Dementia Questionnaire for People with Learning Disabilities

DS: Down syndrome

DSCR: Down syndrome Critical Region

DYRK1A: Dual-Specificity Tyrosine Phosphorylation-Regulated Kinase 1A

EDTA: Ethylenediaminetetraacetic acid

EF: Executive functions

EGCG: Epigallocatechin-3-gallate

FBS: Fetal bovine serum

fT_4: free thyroxine

GSK-3β: Glycogen synthase kinase 3 β

GTC: Green Tea Catechins

Hcy: Homocysteine

HDLc: High-density lipoproteins cholesterol

ID: Intellectual disability

iPSCs: induced pluripotent stem cells

IQ: Intellectual quotient

K-BIT: Kaufman 30 Brief Intelligence Test

LDLc: Low-density lipoproteins cholesterol

MeDi: Mediterranean Diet

MCI: Mild Cognitive Impairment

MMSE: Mini Mental State Examination

MTHFR: methylenetetrahydrofolate reductase

MTR: 5-methyltetrahydrofolate-homocysteine methyltransferase

MTRR: 5-methyltetrahydrofolate-homocystene methyltransferase reductase

NQO1: NAD(P)H Quinone Dehydrogenase

oxLDL: oxidized-LDL

OM: Olfactory Mucosa

ONPCs: Olfactory Neuronal Precursor Cells

ORNs: Olfactory Receptor Neurons

p: p-value

PIB: Pittsburgh compound B

p-tau: phosphor-tau

PS1: Presenilin-1

R: Pearson correlation

rho: Spearman correlation

SAH: S-adenosyl-homocysteine

SAHH: SAH hydroxylase

SAM: S-adenosyl-methionine

sAPPα: soluble APPα

sAPPβ: soluble APPβ

SCS: DMR Sum of Cognitive Scores

SD: Standard Deviation

SE: Standard Error

SOS: DMR Sum of Social Scores

SSP: Spatial Span

SVFT: Semantic Verbal Fluency Task

TC: Total Cholesterol

ToLDx: Tower of London from Drexel University

t-tau: total-tau

VLDL: Very low-density lipoproteins

YoE: Years of Education

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1. GENERAL INTRODUCTION

1.1. Biomarkers and their association with cognitive dysfunction: importance in Intellectual Disabilities

Biomarkers are understood as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (14). As such, the use of biomarkers is crucial in the context of clinical trials to monitor the safety and efficacy of the treatment. Some biomarkers are well-established and present in clinical practice, and in specific cases they reflect or predict a known biological process. However, despite the plethora of known or proposed pathogenic proteins and mechanisms, only a small number are currently implemented in molecular pathologic subtyping or in biomarker development. In fact, few biomarkers can be objectively measured and evaluated as indicators of normal biological or pathogenic processes, or of pharmacological responses to a therapeutic intervention. Biomarkers also contribute to the knowledge on clinical pharmacology and provide with a basis to the design of clinical trials that expeditiously and definitively evaluate safety and efficacy. Thus, as new treatments appear there is a need to find new biomarkers that are adequate to monitor the target mechanism.

The term "biomarker" covers a wide range of physical measurements and can be obtained from different sources. There are central biomarkers which are obtained from the source of the biological process, or surrogate biomarkers obtained from a peripheral source. In

both clinical trials and clinical practice it is of vital importance that the source of these biomarkers is of easy access and requires a minimally invasive intervention. Especially, in studies related to neurological processes, such as intellectual disabilities, and upon long interventions. For this type of studies, blood and CSF (cerebrospinal fluid) biomarkers are used to disentangle the biological processes underlying neurological diseases. In the case of longitudinal studies and intellectual disabilities obtaining CSF can be problematic, and therefore blood-based biomarkers are preferred.

The identification of new potential biomarkers for the use in clinical trials seeking to improve cognitive function requires a deep understanding of the associations between peripheral biomarkers and cognition. In the case of intellectual disabilities (ID), there is a plethora of genes involved in its etiology, but the mechanisms by which these candidates regulate cognitive function remain poorly understood. Moreover, as there is no previous knowledge on how specific genetic or environmental factors will alter peripheral biomarker associations with cognition, there is a need to compare them to what has been previously described for general population.

1.2. Cognitive profile in Down syndrome

Down syndrome (DS) is the most prevalent genetic form of ID. Its origin is found in the triplication of the chromosome 21 (1). However, cases of partial trisomies have shown that not all the genes in the chromosome have the same contribution, and one or more regions containing genes or non-coding regions relevant for the ID phenotype have been identified and are known as Down syndrome Critical

Regions (DSCR) (15). Nevertheless, the finding of subjects with DS phenotype with partial trisomies that do not include this region has challenged its definition, establishing it as a "susceptibility region" rather than a critical one, and leading to the understanding that the DS phenotype is caused not only by genomic regions triplicated in the chromosome 21, but also by those deregulated in other chromosomes (16). The present work has focused in two genes of the DSCR (*DYRK1A, APP*) two major candidate genes involved in the DS cognitive phenotype that may provide biomarkers related to them.

Individuals with DS have IQ values ranging between 30 and 70, with the mean being 50 (1, 4). This ID arises from deficits in learning, memory, executive functions, and language, which have an impact on adaptive behavior, including a range of everyday skills (4, 17-19).

Individuals with DS demonstrate a consistent pattern of weaknesses in the processing of verbal information relative to visual information. Particularly, children with DS show positive improvement with time in nonverbal cognitive abilities, whereas verbal abilities tends to decelerate throughout adolescence and into adulthood (4).

Longitudinal studies through middle adulthood reveal cognitive declines with increasing age. These declines and the ones seen in late adulthood are frequently associated with a neuropathology of the Alzheimer type (4).

1.2.1. Deficits in Executive Functions and their repercussions on daily living

Executive function (EF) is the cognitive domain required in order to focus in a specific task, solve problems, and adapt to changing situations (2, 3). It is usually divided in three main subdomains: inhibitory control, working memory, and mental flexibility. Inhibitory control includes the ability to inhibit impulses, to pay attention, and to self-control. Working memory is divided in verbal working memory and spatial working memory, and it is understood as the ability of a subject to mentally manipulate information. Finally, mental flexibility is defined as the capacity to adapt your responses to the changing environment. Needless to say, all these capacities are vital to daily living (3).

In individuals with DS, EF is severely impaired both compared to individuals of similar age and to individuals with other intellectual disabilities (2, 3). Such deficits include difficulties in inhibitory control, working memory, and mental flexibility (20). Poor response inhibition emerges in toddlers and continues through adulthood, being more striking on verbally mediated inhibition than on visually mediated ones (4). Many EF deficiencies are subsequent to impairments in other cognitive domains like memory and language. It is the case of the impairment in verbal working memory (16) and in verbally-mediated mental flexibility, where deficits are influenced by the limitations in language; and of the impairment in spatial working memory, where deficiencies in the memory storage capacity play a part. These limitations in EF translate into problems in learning and daily life

functionality, which are reflected on the worse basic adaptive skills individuals with DS have with respect to individuals with other ID (4, 21).

1.2.2. Cognitive deficits associated with age, the AD-like phenotype

Individuals with DS are at a higher risk of Alzheimer disease (AD) than the general population (4). In fact, while the prevalence of AD in control population older than 65 years of age is an 11% (22), in population with DS of the same age the prevalence is between 50 and 75% (4, 22, 23). As for general population, the prevalence of AD increases with age, being an 8% between 35 and 49 years, and increasing to 55% in the fifth decade of life (17, 23, 24). The typical age of onset of the dementia is considered to be between 48 and 56 years of age (24), nevertheless, some individuals never develop AD (1, 17, 22). This variability is likely the manifestation of various sample characteristics within each study, the methodology used, and the time period in which the studies were conducted. For example, apolipoprotein E (ApoE) genotype also increases the prevalence of AD, even at this at risk population (17, 23). Interestingly, and in relation to the previous point, one of the first cognitive domains affected by the decline is EF (2, 4). One of the main challenges in clinical studies in individuals with DS is differentiating the age-related decline from progressive cognitive degeneration associated with dementia. Most individuals with DS have neuropathological changes associated with AD. In some cases, impairments in EF (attention and planning) are associated with the emergence of dementia. Other

symptoms associated with changes in the frontal lobes (apathy, depression, impaired adaptive functioning, and reduced communication) may indicate underlying neurodegeneration secondary to AD (4).

1.3. Biomarkers in Down syndrome

As stated above, the goal of biomarkers is to provide direct evidence of an underlying pathologic process, indirect of a pathologic process through proof of synapse dysfunction and neurodegeneration, or of treatment efficacy. Unfortunately, there is a scarcity of data regarding biomarker changes in adults with normal cognition. In DS although there are no established biomarkers related to cognition, there are some potential ones that could be useful due to the particular genetic and phenotypic characteristics of this population. Such biomarkers include those that have been associated with cognition in healthy population, but may be differentially affected in the DS population. Specific examples are homocysteine (Hcy), peripheral amyloid peptides, lipids, and thyroid hormones. Their characteristics in DS and their relationship to genetics and other factors are detailed below.

Additionally, other non-specific biomarkers have been explored, in particular the Olfactory Neuronal Precursor Cells (ONPCs).

1.3.1. Homocysteine

Homocysteine (Hcy) is a sulphur-containing amino-acid formed during the intracellular conversion of methionine to cysteine in the One Carbon Metabolism (25). Its accumulation when it is not converted to cysteine or methionine has been associated to

cardiovascular diseases and AD (26). Homocysteine leads to the accumulation of low-density lipoprotein, resulting in damage to the blood vessel walls. Increased blood homocysteine levels are correlated not only with cardiovascular disease but also with the severity of cognitive damage and dementia. Additionally, specific inverse associations between high concentrations of Hcy with tasks involving EF and with functionality in activities of daily living have also been described (27-35).

Interestingly, plasma levels of Hcy, methionine, S-adenosylhomocysteine, and S-adenosylmethionine are all significantly decreased in individuals with DS (36). Several causes can explain this reduction. First, the gene for cystathionine beta synthase (CBS) is located on chromosome 21 and is overexpressed in DS (36). Hcy may undergo condensation with serine to form cystathionine, which is catalyzed by CBS, and a 157% increase in CBS enzyme activity has been previously documented in individuals with DS and has been associated with reduced levels of Hcy (37, 38).

Another gene which overexpression in DS has been correlated with decrease of Hcy level is *DYRK1A* (Dual-Specificity Tyrosine Phosphorylation-Regulated Kinase 1A). *DYRK1A* is located in the DSCR and is considered a strong candidate for DS cognitive impairment (6). In fact, the overexpression of this gene has shown to recapitulate part of the DS phenotype, including behavioral alterations that mirror part of the deficits seen in DS, such as spatial learning and cognitive flexibility alterations (5, 6). Moreover, the functional deletion of one of the copies of this gene shows a clear phenotype with

microcephaly, ID, and epilepsy (39), highlighting the importance of this gene in neural development as well as its dosage-sensitivity (6).

DYRK1A is broadly expressed during brain development and its expression levels decrease over time, being confined, in adulthood, to specific regions (6, 40, 41). During the neurodevelopment, DYRK1A is implicated in the proliferation of neuronal progenitors and cell cycle exit, neuronal differentiation, neurogenesis and dendritogenesis, and synaptic plasticity (40). In animal models the overexpression of DYRK1A leads to a decreased proliferation of neuronal progenitors coupled to a premature differentiation which depletes said progenitors leading to a reduced number of neurons (40). Additionally, an extra copy of this gene has also been shown to alter neuritogenesis and spinogenesis as well as synapse formation (40, 42, 43).

However, DYRK1A overexpression has proven to have an important role in the cognitive phenotype. Current research suggests that overexpression of DYRK1A may be a significant factor leading to cognitive deficits in both people with DS and AD, as it also plays a key role in neurodegeneration. In fact, in DS mouse models, *Dyrk1A* is a dosage-dependent gene and its overdosage is sufficient to recapitulate the DS cognitive and neuronal phenotypes (5, 6). Accordingly, normalization of its expression levels rescues motor and learning and memory phenotypes in both transgenic mice overexpressing only Dryk1A (TgDyrk1A) or trisomic Ts65Dn mice (44, 45). Of interest for therapy, normalizing Dyrk1A kinase activity recovers the cognitive phenotypes, and reverses brain defects and dendritic pathology. DYRK1A is also known to phosphorylate tau at the Threonine 212 position, among other positions (43), priming it for

GSK-3β phosphorylation (46) and leading to the formation of neurofibrillary tangles (9, 47). Additionally, DYRK1A is also able to affect the alternative splicing of tau, with its overexpression decreasing the inclusion of the exon 10 of the tau protein, thus, increasing the synthesis of 3R-tau altering the tau isoforms balance and allowing more 3R-tau to be phosphorylated (9). The other protein related to AD, APP (amyloid precursor protein), also happens to be phosphorylated by DYRK1A, and is encoded by chromosome 21 and overexpressed in DS. In this case the phosphorylation enhances the cleavage of APP by β and γ-secretases (8, 9), the full repercussion of this cleavage will be discussed further on as APP is also triplicated in DS (9, 17). Besides, DYRK1A overexpression can be found in brains of patients with AD, strengthening its role in neurodegeneration (48).

DYRK1A also affects Hcy levels, as since it increases NAD(P)H Quinone Dehydrogenase (NQO1) activity, altering the equilibrium in the One Carbon Metabolism, and leading to a depletion of Hcy (49, 50) **(Figure 1)**. Hcy has already been established as a surrogate biomarker of DYRK1A activity, because its concentrations correlate with DYRK1A levels in animal models (10), and with treatment with DYRK1A inhibitors, such as epigallocatechin-3-gallate (EGCG), a flavonol from green tea, in clinical trials (10, 13) (**Annex 1**).

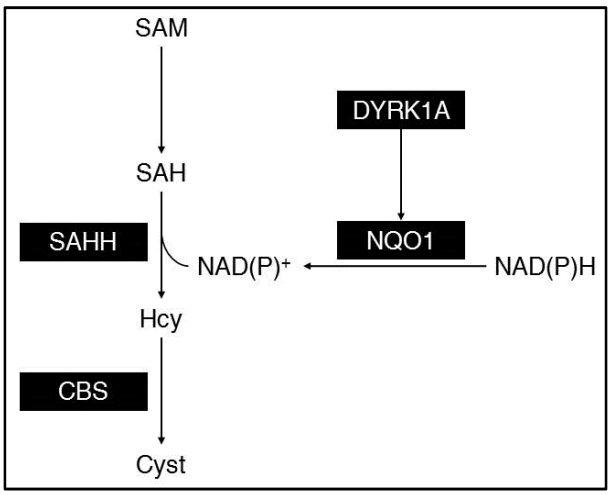

Figure 1: Hcy metabolism. SAM: S-adenosyl-methionine, SAH: S-adenosyl-homocysteine, SAHH: SAH hydroxylase, CBS: Cystathionine-β-synthase, Cys: Cysteine, NQO1: NAD(P)H Quinone Dehydrogenase, Cyst: Cystathionine.

As with other biomarkers, there are external factors that affect Hcy concentrations. One of them is sex, with females having lower concentrations of Hcy than men (51, 52), although some studies report these differences to be non-significant (53, 54). Age can also affect Hcy concentrations, with its concentrations increasing with age either in both sexes (52) or only in females (55). Furthermore, due to Hcy part in One Carbon Metabolism, concentrations of folic acid and B vitamins (B6 and B12) can modify its concentrations, as do polymorphisms for enzymes in this cycle (56-60). Further details can be found in **Chapter II**.

1.3.2. APP, AD risk, and peripheral amyloid biomarkers

Another important gene that affects cognition in individuals with DS is *APP*, which overexpression is thought to be related to the AD-like neuropathology (61). The association between the overexpression of

APP and the higher risk of AD in DS was done following two observations. On one hand, that some familiar forms of early onset AD are caused by the duplication of APP (61), and, on the other hand, by the discovery of an elderly individual with DS who died without amyloid deposition and was found to have a partial triplication of Chromosome 21 that did not include *APP* (7).

The overexpression of APP leads to an overproduction of amyloid-β peptide (Aβ) monomers through the sequential cleavage by β- and γ-secretases (23) **(Figure 2)**. As previously explained, DYRK1A phosphorylates APP favoring the amyloidogenic processing of the protein (8). Furthermore, it has also been proven to phosphorylate the protein of the γ-secretase complex presenilin-1 (PS1) increasing its activity (62). This excess of soluble Aβ, particularly $A\beta_{42}$, accumulates in the brain of subjects with DS from early childhood (62), building up to form amyloid plaques from 40 years of age (63). Interestingly, the overexpression of APP in a trisomic context has also been related to alterations in proliferation, neurogenesis, and neurite length (64-66), having, thus, a potential role on the ID phenotype.

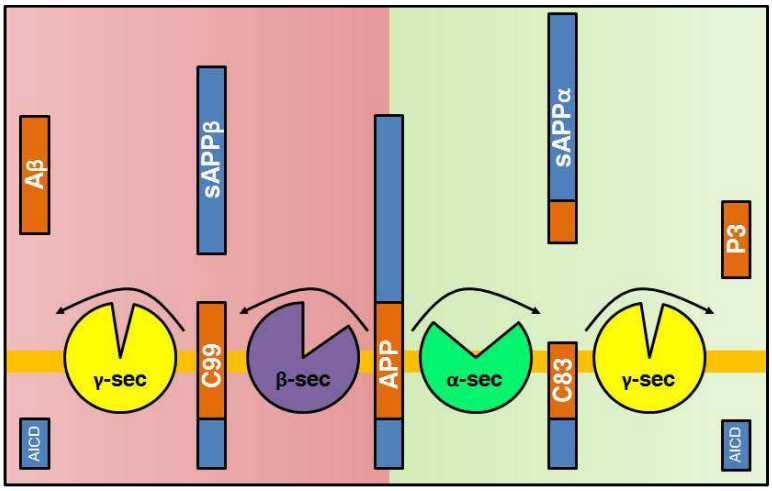

Figure 2: APP metabolism. Red background indicates amyloidogenic processing; green, amyloidogenic. APP: amyloid precursor protein, sAPPα: soluble APP α, AICD: amyloid precursor protein intracellular domain, sAPPβ: soluble APP β, Aβ: amyloid β peptide, γ-sec: γ-secretase, β-sec: β-secretase, α-sec: α-secretase.

In general population the association between peripheral Aβ concentrations and dementia risk is unclear (67). Some studies show that lower $A\beta_{42}$ concentrations and $A\beta_{42}/A\beta_{40}$ ratio are risk factors for dementia development and conversion from mild cognitive impairment (MCI) to AD (68-70). In fact, plasma $A\beta_{1\text{-}42}$ concentrations have been found to inversely correlate with PIB (Pittsburgh compound B) retention, although no differences in the peripheral concentrations were observed depending on diagnosis (71). However, at least one study has found increased $A\beta_{42}$ concentrations in MCI and AD individuals compared to controls (72). With regards to $A\beta_{40}$, its associations with dementia are less clear, although an increase in its concentrations has been previously correlated with MMSE (Mini Mental State Examination) scores (69).

Even though if the association between peripheral amyloid concentrations and cognition is unclear, there are other factors known to predict or affect AD risk and that could be having an effect on Aβ

concentrations. Needless to say, one of the main risk factors to develop spontaneous AD is age (73), and age has been associated to increases in concentrations of Aβ (71, 74). Finally, females are at a higher risk of AD once menopause has started, this risk is multiplied in carriers of ApoE ε4 (73). However, not many studies have focused on the differences in plasma concentrations between genders, with some reporting no differences (75), while others found higher $A\beta_{42}$ concentrations in males (76).

In DS the association between peripheral amyloid concentrations and AD risk is more straightforward. This is possibly due to the increased concentrations of $A\beta_{40}$ in individuals with DS when compared to age-matched controls (77, 78), and to the increased $A\beta_{42}$ concentrations in older populations with DS (79-82). Extensive evidence shows the usefulness of these peripheral biomarkers to assess dementia risk in older DS population (83-85). Interestingly, peripheral $A\beta_{42}$ concentrations have also been correlated to early signs of cognitive decline in young adults with DS (86) **(See Chapter III).**

As previously mentioned, ApoE genotype is a risk factor for dementia in DS. Studies in general population have established that ε4 carriers have between a 3 and 5-fold risk increase, while ε4 homozygotes have between a 15 and 20-fold risk increase. On the other hand, the ε2 allele is considered to be a protective allele against AD development (73, 87). The reason for this increased risk of AD is that the ε4 allele has higher affinity for Aβ monomers leading to a larger amyloid deposition in the brain (73). In the case of plasma amyloid concentrations, the effect of ApoE is not evident. While in young populations no effect of the genotype was found (75), in older

populations there seems to be an association between the ε4 allele and higher concentrations of $A\beta_{40}$ (88).

1.3.3. ApoE and lipid profile

However, the association of ApoE genotype and AD risk is not limited to its role in central amyloid clearance as it is also involved in peripheral lipid concentrations. The carriers and homozygotes of the ε4 allele are usually described to have a worse lipid profile with ε4 homozygotes having higher Total Cholesterol (TC) while ε2 homozygotes have lower concentrations (89, 90), due to the function of ApoE in the peripheral transport of lipids. ApoE circulates in blood associated with chylomicrons, very low-density lipoproteins (VLDL), and LDLc (low density lipoprotein cholesterol), promoting the clearance of triglyceride-rich lipoproteins and decreasing the TC concentrations in the bloodstream (91).

1.3.4. Lipid profile

Few decades ago individuals with DS were considered to be protected from cardiovascular disease, however this finding was challenged by posterior research (92), and for this reason several studies have explored the particular characteristics of the lipid profile in this population. Studies comparing peripheral lipid concentrations between individuals with DS and individuals with other ID or age-matched controls show an altered lipid profile in DS (89, 92-95).

DS and ApoE genotype can alter peripheral lipid concentrations, however they can also be modified by other factors. One of the most clear differences is between males and females, with premenopausal

females having generally a better lipid profile with higher HDLc (high density lipoprotein cholesterol) concentrations, and lower LDL and triglycerides concentrations (89, 95). Age also has an effect, as there seems to be an increase in TC concentrations up to the fifth decade of life and a linear increase in triglyceride concentrations throughout lifetime (89). Additionally, Mediterranean Diet (MeDi) compliance has shown to improve the lipid profile (96, 97).

The alteration in lipid profile seen in DS could be having an effect on cognitive decline, as lipid profile has been shown to be associated to dementia risk in general population independently of ApoE (98-103). Additionally, particular components of the lipid profile have been associated to distinct cognitive domains including IQ (98-100, 104-106). The specific associations between lipid profile and cognition will be explained in detail in **Chapter I**. Additionally, hyperthyroidism has been associated to higher oxLDL (oxidized LDLc) peripheral concentrations, while both hypothyroidism and hyperthyroidism are associated to higher oxidative stress (107).

Thyroid dysfunction is one of the more prevalent alterations present in DS. Its prevalence is between 3 to 54% and it increases with age. Additionally, females also tend to have higher prevalence of it (107). Most of the affected individuals present hypothyroidism -congenital, primary, or subclinical-, while others present hyperthyroidism. The prevalence of autoimmune thyroiditis is also elevated in this population (108).

This alteration of the thyroid function is of special interest in DS as hypothyroidism is known to cause intellectual disability in children (108).

1.4. Olfactory Neuronal Precursor Cells

One of the main limitations of using peripheral biomarkers in neuropsychopharmacology is the lack of direct readouts of the impact a treatment is having on the neurons in the brain. To solve this problem, efforts have focused on obtaining neuronal cells from peripheral tissues and one of the most accessible and promising sources is the olfactory mucosa (OM).

The OM is found in the neurolfactory epithelium of the cribriform plate, and the superior and middle turbinate (109). Due to the nature of the tissue and its high exposure to noxious agents, the olfactory receptor neurons (ORNs) die and are replaced by a process of neurogenesis that is maintained through the lifespan (110). This neurogenesis relies on a population of multipotent stem cells, or ONPCs, present in the neuroepithelium of the OM that can give rise to both neuronal and non-neuronal cells (111, 112). The capacity of proliferation of the ONPCs has made them the perfect model to study a wide array of neurological diseases *in vitro* (112).

1.5. The use of biomarkers for DS therapeutic trials

DS is considered to be an orphan disease, as there is no treatment to help improve ID, its most prevalent characteristic. In these last years, efforts have been multiplied in the search of a treatment that could

ameliorate the cognitive impairment. *In vivo* studies have explored the capacities of all kind of compounds, from antioxidants to neurotransmitter modulators, and some of these studies have even been translated into clinical trials. Of special interest is the treatment using Epigallocatechin-3-gallate (EGCG) (113-115).

1.5.1. EGCG

EGCG is the most abundant catechin in green tea (12). Its properties have been extensively studied in neurodegeneration and AD and have been attributed to its antioxidant effects, its anti-amyloidogenic and anti-neuroinflammatory effects, among others (116) **(See Annex 2)**.

One of the reasons for using EGCG in DS is the specific capacity of the catechin to interact and modulate DYRK1A kinase activity. A first study by Adayev *et al* (117), described the potential of EGCG to bind and inhibit DYRK1A *in vitro*. In light of these results, and taking into account the role of DYRK1A in DS, several *in vivo* studies followed. EGCG rescued the brain morphogenesis and learning alterations seen in transgenic mice overexpressing DYRK1A (11). Experiments in the trisomic mouse model Ts65Dn support the rescue of the behavioral phenotype in DS (118), which allowed the translation into clinical trials where EGCG in combination with cognitive stimulation had a positive impact on some specific tasks involving executive functions (13, 118).

In a Phase II clinical trial (13), changes in peripheral biomarkers reported in this Thesis were detected **(See Annex 1)**. Upon EGCG treatment, Hcy concentrations increased, and returned to baseline levels after treatment discontinuation. On the other hand, EGCG

modified the concentrations of several components of the lipid profile including total TC, HDLc, and oxLDL.This potential to modify the lipid profile has also been observed in several other studies (119) and will be further explained in **Chapter I**.

2. HYPOTHESIS AND OBJECTIVES

2.1. Hypothesis

We hypothesize that specific plasma biomarkers associated with executive function in other populations, may be useful, but present specific characteristics in DS. We have separately analyzed the following:

2.1.1. Study I: Lipid profile and Executive Functions

Although lipid profile has long been known to influence cognition in general population, its effect on cognition in DS is not known, despite an altered lipid profile having been detected in this population. We postulate that lipid profile can be used as a biomarker of cognitive function and cognitive decline in DS. Furthermore, we hypothesize that EGCG can have an effect on the association between lipid concentrations and executive function.

2.1.2. Study II: Homocysteine and Executive Functions

Individuals with DS have decreased homocysteine (Hcy) concentrations. Hyperhomocysteinemia is associated with worse executive functions in the general population. Our hypothesis is that, Hcy concentrations in our population will also be associated to executive functions. Additionally, we postulate that Hcy concentration changes can be used as a biomarker of treatment-induced cognitive change.

2.1.3. Study III: Years of education and markers of cognitive decline

Alzheimer-like disease is highly prevalent in DS, due to the overexpression of APP. Our main hypothesis is that plasma concentrations of amyloid peptides are associated with early signs of dementia and can be modified through external factors such as education.

2.1.4. Study IV: Potential use of ONPCs as biomarkers

Peripheral samples, such as blood and skin, have been used for decades in psychiatric research as surrogates for central nervous system. Many primary cells in blood samples tend to be highly differentiated, and they may lack many of the signaling pathways and biological processes that are present in the brain. Even shared signaling pathways might function differently in different biological contexts. Thus, peripheral biomarkers in neuropsychopharmacology have limitations as they do not allow the direct observation of the processes happening in neuronal cells. Our proposal is that neuronal precursors obtained from the nasal neuroepithelium could be potentially used as direct biomarkers of neuronal function.

2.2. **Objectives**

2.2.1. General objective

The main objective of this thesis is to assess the utility of peripheral biomarkers in terms of their association to executive function and cognitive decline. Furthermore, we will also focus on the exploration of new potential sources of biomarkers.

2.2.2. Study I

This study focused on analyzing the association between specific tasks and functional measures related to executive functions and cognitive decline, and different components of the lipid profile. Moreover, we aimed to investigate how age, sex, ApoE genotype, and MeDi compliance could be affecting such associations.

Additionally, we analyzed the effect that treatment with EGCG had on the associations between these variables.

2.2.3. Study II

In this study we have focused on studying the associations between executive functions and homocysteine concentrations taking into account possible confounders such as age, sex, MeDi compliance, or genetic polymorphisms.

As in the first study, the longitudinal effects of EGCG will also be taken into account in order to evaluate the usefulness of homocysteine as a biomarker of cognitive change.

2.2.4. Study III

Our aim was to explore the associations between plasma amyloid concentrations and early signs of cognitive decline, as well as the potential effects of ApoE genotype on any of the variables. Furthermore, we wanted to assess the effects that educational length has in cognitive decline markers: biochemical, cognitive, and functional.

2.2.5. Study IV

Finally, this study was focused on assessing the use of nasal exfoliates and ONPCs as source of biomarkers. Additionally, we also examined the effect of serum deprivation on protein expression and morphology of the ONPCs.

3. CHAPTER I: Lipid profile and Executive Functions: Basal and Longitudinal Associations.

3.1. Introduction

As stated in the **General Introduction** a number studies have evaluated the associations between the lipid profile and cognition in the general population. Such associations can be detected throughout lifespan and, in some cases, lipid concentrations have been proposed as risk factors of cognitive decline. Studies in individuals of almost all age groups have demonstrated that higher concentrations of total cholesterol, LDLc, or triglycerides are associated to lower IQ scores (98, 99, 104, 105), while high HDLc concentrations have the opposite effect and correlate with better intelligence (99, 105). These effects are not limited to IQ, as higher concentrations of cholesterol have been correlated with worse memory and learning in middle age (100). Memory and a better performance in EF, specifically in working memory, have also proven to be directly correlated with HDLc concentrations (98, 106).

An altered lipid profile is a risk factor for cognitive decline and AD. This altered lipid profile would include both high TC and triglycerides concentrations (98-101), with the former being also linked to MCI (100). For LDLc concentrations the effect on dementia risk is less clear (100) with some studies observing a U-shaped effect in elderly males (102), while others observe an inverse correlation between LDLc concentrations and MMSE score in AD patients (120). This ambiguous relation was also observed for the oxidized form of LDLc,

with no association seen with its concentrations and dementia risk (103). On the other hand, higher HDLc concentrations are the only ones that are thought to have a protective effect against cognitive decline (100, 106, 121).

ApoE polymorphism is a known modifier of the lipid profile. Carriers of the ε4 allele have higher concentrations of both TC and LDLc than the ε3 homozygotes, while ε2 carriers have lower concentrations than the ε3 homozygotes (105, 122). For triglycerides, however, ε3 homozygotes have lower concentrations than the carriers of the other two alleles (105). Furthermore, the ApoE allele ε4 is a risk allele for the development of AD (73, 87) and has been associated to cognition. Female subjects carrying either ε2 or ε4 alleles have a higher IQ than ε3 homozygotes (105). In elderly population, ε4 carriers had worse cognitive performance than the non-carriers, and there was an interaction between ApoE genotype and HDLc concentrations, with ε4 non-carrier subjects with higher HDLc having better cognitive scores (122).

Another extrinsic factor affecting lipid concentrations is diet. In this case, MeDi is known to reduce risk for cardiovascular diseases and to decrease TC and LDLc concentrations while increasing HDLc (96, 97). Additionally, MeDi is known to be protective against the development of AD (123), and an interventional study has also proven its capacity to improve cognitive function in elderly population (124).

Individuals with DS have an altered lipid profile. In fact, a comparison between children with DS and control siblings showed that children with DS have higher triglycerides, total cholesterol and LDLc, and

lower HDLc serum concentrations (93). Additionally, adults and children with DS have higher oxLDL concentrations than age-matched individuals in the general population (94). Studies comparing individuals with intellectual disabilities and individuals with DS from the same institutions found that subjects with DS had higher triglycerides (125) and VLDL (126) concentrations than the individuals with other ID.

Despite this alterations in lipid profile and the fact that it has proven to affect cognition, few studies have looked into the associations between cognition and lipid profile in individuals with DS, with only one study reporting high concentrations of cholesterol to be a risk factor for AD in middle aged adults with DS (127).

Extensive research has analyzed the effects of green tea and GTC (green tea catechins) on lipid profile in a wide-range of populations. Most of the studies agree that consumption of green tea or GTC decreases LDLc concentrations (119, 128-132). Additionally, some also found decreases in TC (119, 129) and oxLDL (133). However, and probably due to the large number of different GTC sources and the diverse diseases tackled, there are other studies that fail to find an effect on peripheral lipid concentrations (134-139). To our knowledge, the only studies that have examined the effect of EGCG on lipid profile in population with DS are the ones performed in our laboratory. In a pilot clinical trial significant differences could be observed between treatment groups for LDLc and TC concentrations (10). In the Phase II clinical trial this Thesis is nested in, significant differences were seen for TC, HDLc, and oxLDL (13) (**see Annex 1**).

3.2. **Methods**

3.2.1. Participants

The individuals included in this study were young adults with DS drawn from the TESDAD study that followed the study inclusion criteria (13) (See **Annex 1**). Subjects who meet criteria for neurological diseases other than DS or had relevant medical disease, co-morbid mental disorder or were undergoing any treatment interfering with cognitive functions, were not included in the trial. Furthermore, individuals under treatment with TC-lowering medication or with a trisomy other than full trisomy were also excluded from this analysis (See **Figure 3** for a Consort Diagram). The study was approved by the clinical research ethics committee. More details of the sample and the methodology can be found in **Annex 1.**

The study was conducted in accordance with the Declaration of Helsinki and Spanish laws concerning data privacy. The protocol was approved by the Ethical Committee of the Parc de Salut Mar of Barcelona (CEIC-PSMAR). Upon arrival at the research centre (Hospital del Mar Medical Research Institute-IMIM), participants, parents and legal guardians (in case of legal incapacitation) were informed of the ensuing protocol and they gave their written informed consent before participating.

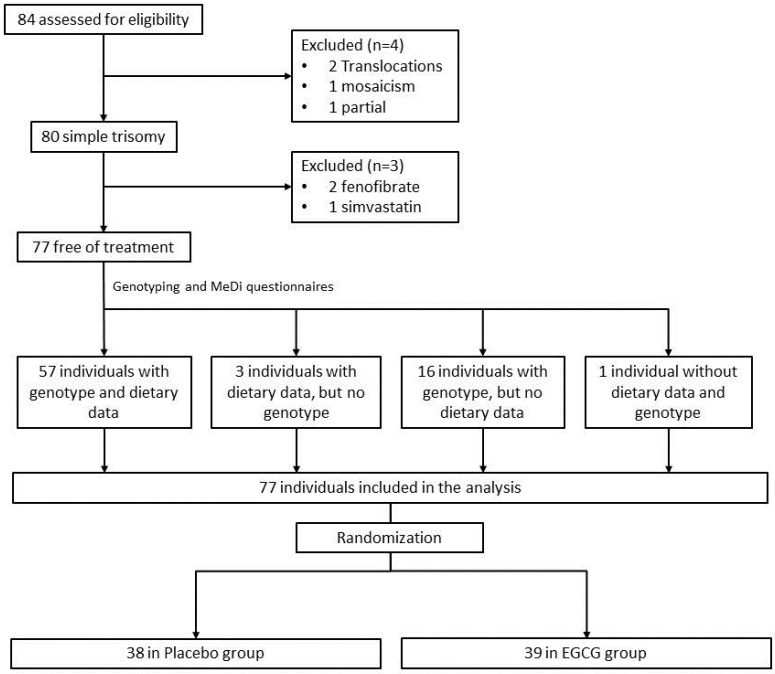

Figure 3: Consort diagram of the population included in this study.

3.2.2. Neuropsychological test battery

The TESDAD Battery (140) was administered and for this study, we selected specific tests related to executive functions. Attention and working memory were assessed with Spatial Span (CANTAB) (141) and Digit Span (142), that evaluated visual and verbal information respectively. Also, other aspects related to executive function were measured with the Semantic Verbal Fluency Task (SVFT) (participants were asked to generate as many words as possible in 1 min belonging to the specified category of animals) (143). The Tower of London from Drexel University (ToLDx) (144) was used to measure planning capacity and The Cats and Dogs Test (145) to assess response inhibition.

Intellectual quotient (IQ) was estimated using The Kaufman 30 Brief Intelligence Test (K-BIT) (146). A more detailed description of the complete neuropsychological battery and references can be found in **Annex 1**.

3.2.3. Adaptive behavior and dementia onset

Adaptive behavior was assessed with the adult version of the Adaptive Behavior Assessment System-Second Edition (ABAS-II) (147). Additionally, we screened for changes related to dementia onset with the Dementia Questionnaire for People with Intellectual Disabilities (DMR) (148). Researchers ensured that caregivers understood how to complete the questionnaire and solved all doubts before and after completion, and verified that all questions were filled. For more details see **Annex 1**.

3.2.4. Mediterranean Diet Compliance questionnaire

MeDi compliance questionnaires were administered to the caregivers, they consisted in a 14-question questionnaire that has been previously validated in the PREDIMED study (149, 150) (See **Annex 3** for an example). No information on MeDi compliance could be recollected from 17 of the 77 individuals **(Figure 3)**.

3.2.5. Plasma lipid concentrations

Blood samples were collected after an overnight fast early in the morning. Blood was drawn into 8mL Heparin Lithium tubes (B&D, UK), centrifuged at 4ºC for 15 min at 3000 rpm, and the plasma was

distributed in aliquots and stored at -70ºC until analysis. Total cholesterol, and triglyceride levels were measured using standard enzymatic methods. HDL-cholesterol was determined by an accelerator selective detergent method (ABX-Horiba Diagnostics, Montpellier, France), in an automated PENTRA-400 analyzer (ABX-Horiba Diagnostics, Montpellier, France). Low density lipoprotein (LDL) cholesterol was calculated by the Friedewald formula whenever triglycerides were <300 mg/dL. Oxidized LDL (oxLDL) concentrations in plasma were measured by a sandwich ELISA procedure using the murine monoclonal antibody mAB-46 as the capture antibody bound to microtitration wells and a peroxidase conjugated anti-apolipoprotein B antibody that recognizes oxLDL bound to the solid phase (oxLDL, Mercodia, Uppsala, Sweden).

3.2.6. ApoE genotyping

Genomic DNA was extracted from the peripheral blood leukocytes of all the participants using Flexi Gene DNA kit (Qiagen Iberia, S.L., Spain) according to the manufacturer instructions. ApoE genotype was determined by PCR using a modified protocol from Yousuf *et al* (151), the following primers were used 5'-GGACGAGACCATGAAGGAGTT-3' (forward) and 5'-GCCCCGGCCTGGTACACTGCCA-3' (reverse), the reaction was carried out in a total volume of 15µL in the following conditions: 1X Green GoTaq Reaction Buffer (Promega, Madison, WI), 1.65 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µmol of each primer, 0.5 U of GoTaq G2 DNA Polymerase (Promega), and approximately 50 ng of genomic DNA. The amplification conditions included an initial denaturation of 7 min at 94ºC, followed by 40 cycles as follows: 30 seconds of

denaturation at 94ºC, 30 seconds of annealing at 64.5ºC and 30 seconds of elongation at 72ºC, the final extension step was of 7 minutes at 72 degrees. The final PCR product was 306bp.

The PCR product was digested in parallel overnight at 37ºC with 5U of AflIII and HaeII (New England Biolabs) using the provided digestion buffers. ε2 allele was cut in two bands of 168 and 138bp by AflIII, and remained uncut (306bp) for HaeII; ε3 allele presented two bands of 168 and 138bp for AflIII, and two bands of 283 and 23bp for HaeII (23bp band was not visible); ε4 allele was uncut by AflIII (306bp) and cut into two bands by HaeII (283 and the invisible 23bp band). The digestion products were resolved by electrophoresis on a 3% agarose gel together with undigested PCR product that was used as reference.

Four of the individuals could not be genotyped.

3.2.7. Data analysis

Results are described by means of measures of both central tendency (mean) and variability (standard deviation and range) for numeric variables, and absolute and relative frequencies for categorical variables. In the case of the IQ, only the median is reported because no distinction is made of values below 40. Because of the distribution of Triglycerides concentrations all the analyses have been performed using the log transformation of the same. ANCOVA models were used to explore the effects of age, sex, ApoE genotype, and MeDi on both cognition and lipid profile. Due to missing values in both ApoE and MeDi compliance, no models were performed combining the two variables. Further pairwise analyses were performed for the ApoE

genotype. Linear models were fitted to study the associations between the different components of the lipid profile or the ratios of the same and cognition.

ANCOVA models were fitted to analyze the effect of treatment in our variables of interest. Models were fitted for age, sex, and ApoE, and were further stratified by sex to analyze the specific effects of the treatment. Linear models stratified by sex and treatment were fitted to study the association between changes in lipid profile and changes in cognition.

Statistical significance was set at 0.05. No correction for multiple testing was performed as it is considered an exploratory study. All statistical analyses were performed using the statistical software package R (Version 3.1.3.; The R Foundation for Statistical Computing, Vienna, Austria).

3.3. **Results**

3.3.1. Descriptive demographics

Our study sample was comprised of a total of 77 young adults with DS, from which ApoE genotype was only available for 73. ANCOVA models adjusted for gender and age were performed to analyze the differences between males and females, frequency differences were evaluated through Chi-square **(Table 1)**. Differences in lipid profile are represented in **Figure 4**. Women had higher K-BIT scores than males. An age effect for the log(Triglycerides) values (Estimate=-0.026, SE=0.011, p=0.018) and for the log(Trig/HDLc) values (Estimate=-0.032, SE=0.013, p=0.015) was observed.

Table 1: Descriptive demographics.

		Total (n=77)	Males (n=39, 51%)	Females (n=38, 49%)	p
Age	Mean (± SD)	23.4±4.3	22.6±3.7	24.2±4. 7	0.09
	Range	16-34	16-31	17-34	
IQ	Median	42	40	44	0.15
K-BIT score	Mean (± SD)	105.1±16.1	101.2±14.9	108.9±16.4	**0.04**
	Range	80-154	80-144	80-154	
Categorical IQ	<40	31 (40%)	17 (44%)	14 (37%)	0.55
	≥40	46 (60%)	22 (56%)	24 (63%)	
ApoE	ε2/ε3	8 (11%)	3 (8%)	5 (14%)	0.56
	ε3/ε3	58 (79%)	32 (84%)	26 (74%)	
	ε3/ε4	7 (10%)	3 (8%)	4 (11%)	
MeDi Compliance	Yes (>7)*	43 (72%)	23 (77%)	20 (67%)	0.39
	No (≤7)	17 (28%)	7 (23%)	10 (33%)	
TC (mg/dL)	Mean (± SD)	173.3±31.3	172.1±29.5	174.5±33.5	0.88
	Range	111-240	111-236	120-240	
Triglycerides (mg/dL)	Mean (± SD)	70.9±28.2	70.8±30.8	71.1±25.7	0.46
	Range	20-175	24-175	20-137	
HDLc (mg/dL)	Mean (± SD)	46.6±9.1	44.3±6.1	49.1±10.8	0.03
	Range	27.3-73.1	27.3-57.9	32.1-73.1	
LDLc (mg/dL)	Mean (± SD)	112.9±27.9	114.6±25.8	111.2±30.2	0.46
	Range	44.9-174.7	44.9-172.2	62.3-174.7	
oxLDL (U/L)	Mean (± SD)	46.7±12.9	47.8±12.6	45.5±13.4	0.4
	Range	12.1-87.3	12.1-85.0	20.7-87.3	
Ratio TC/HDLc	Mean (± SD)	3.82±0.89	3.94±0.76	3.69±1.00	0.26
	Range	1.99-6.87	1.99-5.81	2.24-6.87	
Ratio Trig/HDLc	Mean (± SD)	1.61±0.79	1.65±0.82	1.56±0.77	0.9
	Range	0.37-4.58	0.57-4.58	0.37-3.39	
Ratio LDLc/HDLc	Mean (± SD)	2.51±0.78	2.63±0.65	2.38±0.88	0.16
	Range	0.81-5.21	0.81-4.18	1.09-5.22	
Ratio oxLDL/LDLc	Mean (± SD)	0.42±0.10	0.42±0.08	0.43±0.12	0.6
	Range	0.14-0.71	0.27-0.55	0.14-0.71	

* refers to score in the MeDi questionnaire. Subjects with scores higher than 7 are considered compliant with the Mediterranean Diet. HDL: high density lipoprotein; IQ: Intellectual Quotient; LDL: low density lipoprotein; oxLDL: oxidized LDL; SD: Standard Deviation; TC: Total Cholesterol, Trig: triglycerides

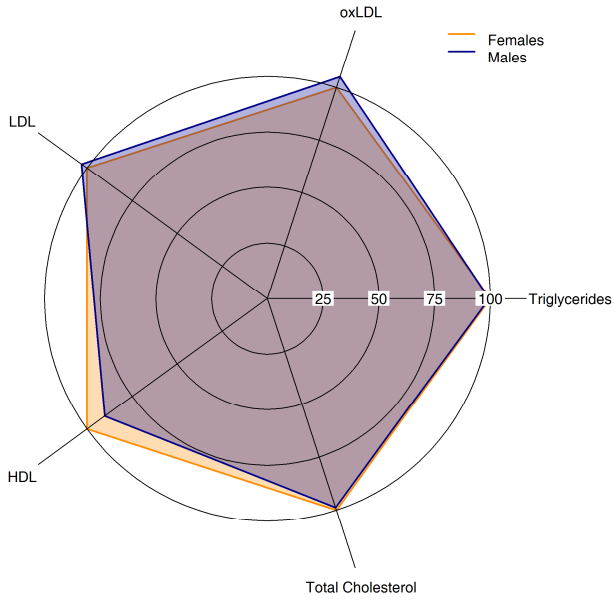

Figure 4: Radar plot representing the differences in lipid profiles between males and females. Mean values in females are taken as reference and assigned as 100%, men had significantly lower HDL concentrations.

3.3.2. Effects of ApoE and MeDi compliance on Lipid profile

ANCOVA models fitted for gender, age, and ApoE genotype were applied in order to analyze the effect of ApoE on the different components of the lipid profile. ε2 carriers had significantly lower concentrations of TC and LDLc as well as lower TC/HDLc and LDLc/HDLc ratios than both ε3 homozygotes and ε4 carriers **(Table 2)**.

Additional models with MeDi compliance, but not ApoE genotype, showed no effects of diet compliance on lipid concentrations.

Table 2: Pairwise comparisons between ApoE genotypes

		ε3/ε3 vs. ε2/ε3	ε3/ε4 vs. ε2/ε3	ε3/ε3 vs. ε3/ε4
TC	Estimate	30.520	57.220	26.701
	p	**0.019**	**0.001**	*0.06*
	95% CI	[4.236,56.804]	[21.335,93.106]	[-1.032,54.433]
log(Trig)	Estimate	0.055	0.301	0.246
	p	0.93	0.31	0.25
	95% CI	[-0.319,0.429]	[-0.194,0.796]	[-0.124,0.616]
HDLc	Estimate	-4.328	-5.254	-0.926
	p	0.4	0.49	0.96
	95% CI	[-12.374,3.718]	[-16.239,5.731]	[-9.416,7.563]
LDLc	Estimate	33.518	56.565	23.047
	p	**0.005**	**0.0002**	*0.06*
	95% CI	[9.102,57.935]	[24.257,88.873]	[-1.114,47.207]
oxLDL	Estimate	9.256	11.859	2.603
	p	0.15	0.18	0.87
	95% CI	[-2.471,20.983]	[-4.129,27.846]	[-9.773,14.978]
TC/HDLc	Estimate	0.925	1.654	0.729
	p	**0.012**	**0.0008**	*0.079*
	95% CI	[0.171,1.679]	[0.625,2.684]	[-0.066,1.525]
log(Trig/HDLc)	Estimate	0.149	0.420	0.271
	p	0.71	0.22	0.32
	95% CI	[-0.304,0.603]	[-0.180,1.019]	[-0.178,0.719]
LDLc/HDLc	Estimate	0.884	1.471	0.587
	p	**0.01**	**0.0009**	0.11
	95% CI	[0.183,1.585]	[0.543,2.399]	[-0.107,1.281]
oxLDL/LDLc	Estimate	-0.039	-0.095	-0.056
	p	0.6	0.18	0.34
	95% CI	[-0.135,0.058]	[-0.222,0.033]	[-0.151,0.039]

p: p-value; CI: Confidence Interval; HDL: high density lipoprotein; LDL: low density lipoprotein; oxLDL: oxidized LDL; TC, total cholesterol; Trig, triglycerides

3.3.3. Effect of ApoE genotype, gender, MeDi compliance, and age on cognitive and behavioral variables

ANCOVA models were fitted in order to understand the effect of gender, ApoE, and age on the studied cognitive variables. Other than the difference in K-BIT scores, females also performed better in the ToLDx task with significantly higher correct movements (Females vs. Males: Estimate=0.921, SE=0.349, p=0.01), and a trend to perform a shorter execution time (Females vs. Males: Estimate=-135.048, SE=71.044, p=0.06). Such results would suggest better planning skills in females. No age effect was detected for any of the variables. ApoE genotype had a significant effect in the SVFT, with ε3 homozygotes having a worse performance than ε2 carriers (Estimate=-4.381, p=0.021, 95% CI=[-8.194,-0.568]), as well as on Cats and Dogs Inhibition Time, with ε4 carriers having significantly longer inhibition times than ε2 carriers (Estimate=20.517, p=0.009, 95% CI=[4.435,36.599]), and non-significantly higher than ε3 homozygotes (Estimate=12.674, p=0.054, 95% CI=[-0.186,25.534]). Although our sample size limits the meaningfulness of these observations, all show a better performance of ε2 in semantic verbal fluency and in inhibitory control.

Further models for MeDi were performed. Individuals in the compliance group had a significantly higher span on the Direct Digit task (Estimate=-0.444, SE=0.210, p=0.039), but this was the only cognitive task were an effect of MeDi could be observed. On functional variables, those in the compliance group had higher scores

in the ABAS-II Self-Care subscale (Estimate=-4.816, SE=1.928, p=0.015) and in the total ABAS-II (Estimate=-51.943, SE=22.072, p=0.02). Non-significant trends were also observed for the ABAS-II subscales Self-Direction (Estimate=-6.619, SE=3.836, p=0.09) and Functional Academics (Estimate=-8.575, SE=4.504, p=0.06). Finally, those in the non-compliance group had higher scores in the Total DMR (Estimate=6.655, SE=1.814, p=0.0006). Individuals in the non-compliant group had lower attention, general functionality, and earlier dementia signs **(Figure 5)**.

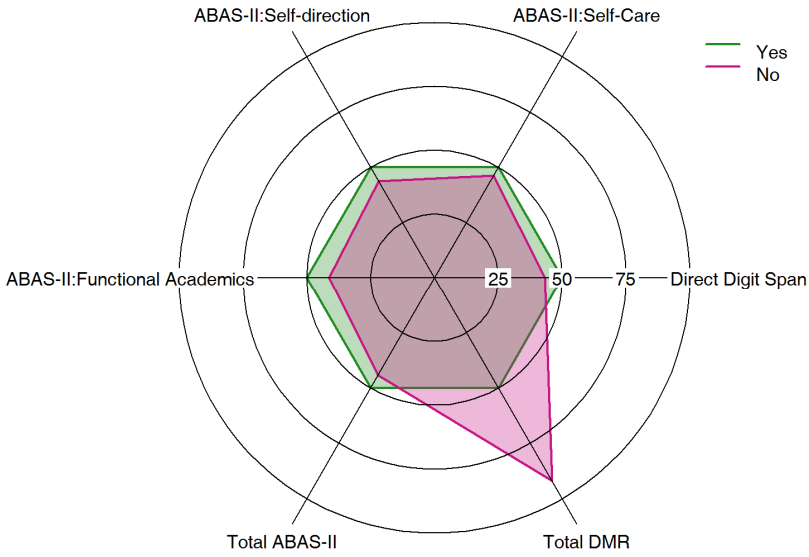

Figure 5: Radar plot representing the differences cognition and functionality in MeDi compliance groups. Mean values in the compliant group ("Yes") are taken as reference and assigned as 100%. ABAS-II: Adaptive Behavior Assessment System Second Edition; DMR: Dementia Questionnaire for People with Learning Disabilities.

3.3.4. Basal associations with cognition

Models for each of the cognitive variables were fitted for HDLc, TC, LDLc, oxLDL, and the logarithm of triglycerides concentrations. Similar models were fitted for the ratios (LDLc/HDLc, TC/HDLc, log(Trig/HDLc), and oxLDL/LDLc). The sample was stratified for gender to take into account the differences in both cognition and the lipid profile that have been previously described. Significant results are described below, but full results can be found on **Supplementary Table 1**.

HDLc showed a gender-dependent association with several tasks. In the case of males, HDLc was directly associated with SSP forward span length, a cognitive variable that also showed a significant inverse association with TC concentrations.

In females, the associations were mainly found in the ToLDx task. Higher concentrations of HDLc were significantly associated to shorter resolution times, as were higher values of log(Trig). Higher concentrations of HDLc were also significantly associated to a higher number of correct movements; similarly, higher LDLc concentrations, higher log(Trig) values, and lower TC concentrations were associated to higher number of correct words. For this same variable an inverse association with the TC/HDLc ratio was observed. A non-significant association was also found between the total number of movements in ToLDx and HDLc concentrations.

Independently of gender HDLc was directly associated to Functional Academics ABAS subscale scores. Such association was also observed with oxLDL concentrations.

Therefore, HDLc concentrations had a direct impact on daily-life functionality while also being associated to better attention and planning skills. The associations with the cognitive tasks were also observed for TC, although in this case higher concentrations lead to worse performances.

Other components of the lipid profile presented associations independently of HDLc, and those associations were also gender-dependent and found only in males. All of the observed associations were unexpected; higher oxLDL concentrations and oxLDL/LDLc were directly associated to SSP Backward Span Length, and higher scores in the Self-care subscale of the ABAS-II were associated to higher TC concentrations and higher TC/HDLc ratio. This same functional variable had an inverse association with the LDLc/HDLc ratio. These associations point to a higher oxidative status being associated to better spatial working memory, and higher TC concentrations to better functionality.

3.3.5. Effects of EGCG on the lipid profile

In the clinical trial, effects of EGCG on the lipid profile have already been described, with the treatment significantly decreasing concentrations of TC, HDLc, and oxLDL (13). Similar analyses were performed with this study subpopulation **(Supplementary Table 2)** and significant differences between the two treatments were found at all time points for LDL **(Figure 9)** and oxLDL **(Figure 10)** concentrations. The TC/HDLc **(Figure 11)** and LDLc/HDLc **(Figure 12)** ratios were only significant in the 6-month, and 3 and 6-months time-points, respectively. Stratified analysis according to sex

shows a clear treatment effect on lipid profile in males, with significantly higher decreases in the concentrations of all the biomarkers at 12-months **(Figures 6 to 12)**, with TC, LDLc, and oxLDL showing effects as soon as the 3rd month **(Figures 6, 9, and 10)**. Contrarily, in females there is not an important treatment effect, as the only significant differences were at 3 and 6-month time-points, with higher decreases in the LDLc/HDLc ratio **(Figure 12)**.

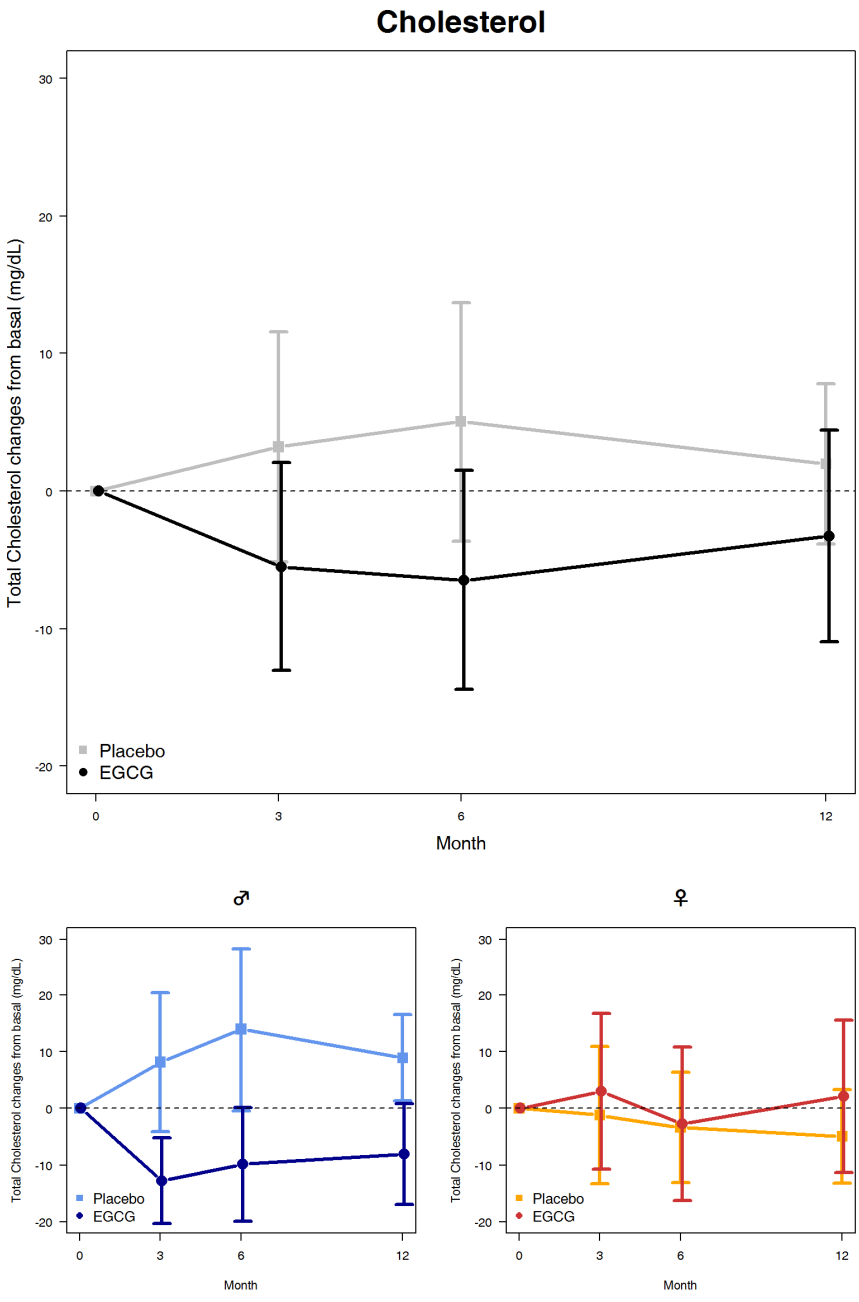

Figure 6: Changes in TC concentrations from baseline. On the top graph changes for the Placebo group are in light grey, while EGCG is in black. On the left bottom panel, stratified changes for males show differences between the EGCG group (dark blue) and the Placebo group (light blue). On the right bottom panel no differences are observed in females between Placebo group (light orange) and EGCG group (dark orange).

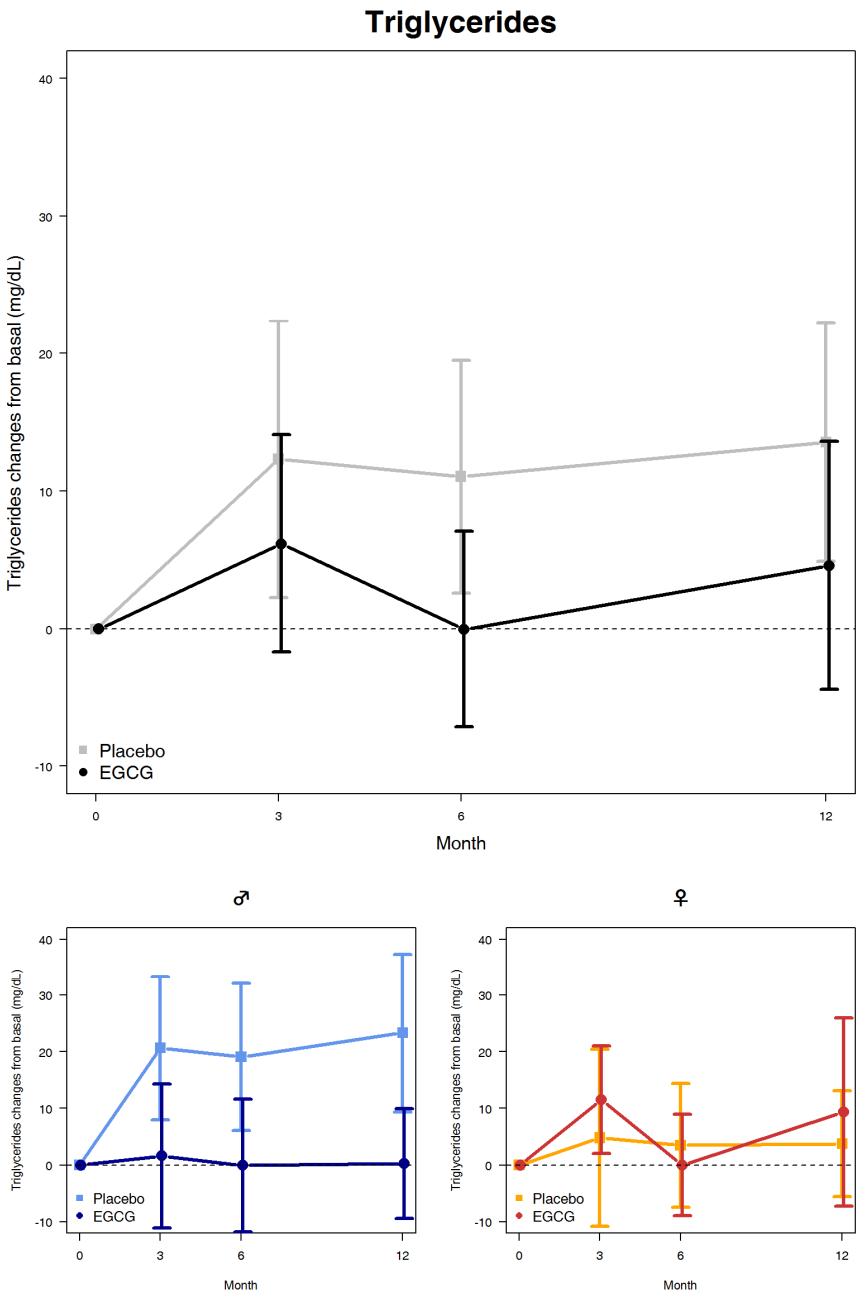

Figure 7: Changes in Tryglicerides concentrations from baseline. On the top graph changes for the Placebo group are in light grey, while EGCG is in black. On the left bottom panel, stratified changes for males show differences between the EGCG group (dark blue) and the Placebo group (light blue). On the right bottom panel no differences are observed in females between Placebo group (light orange) and EGCG group (dark orange).

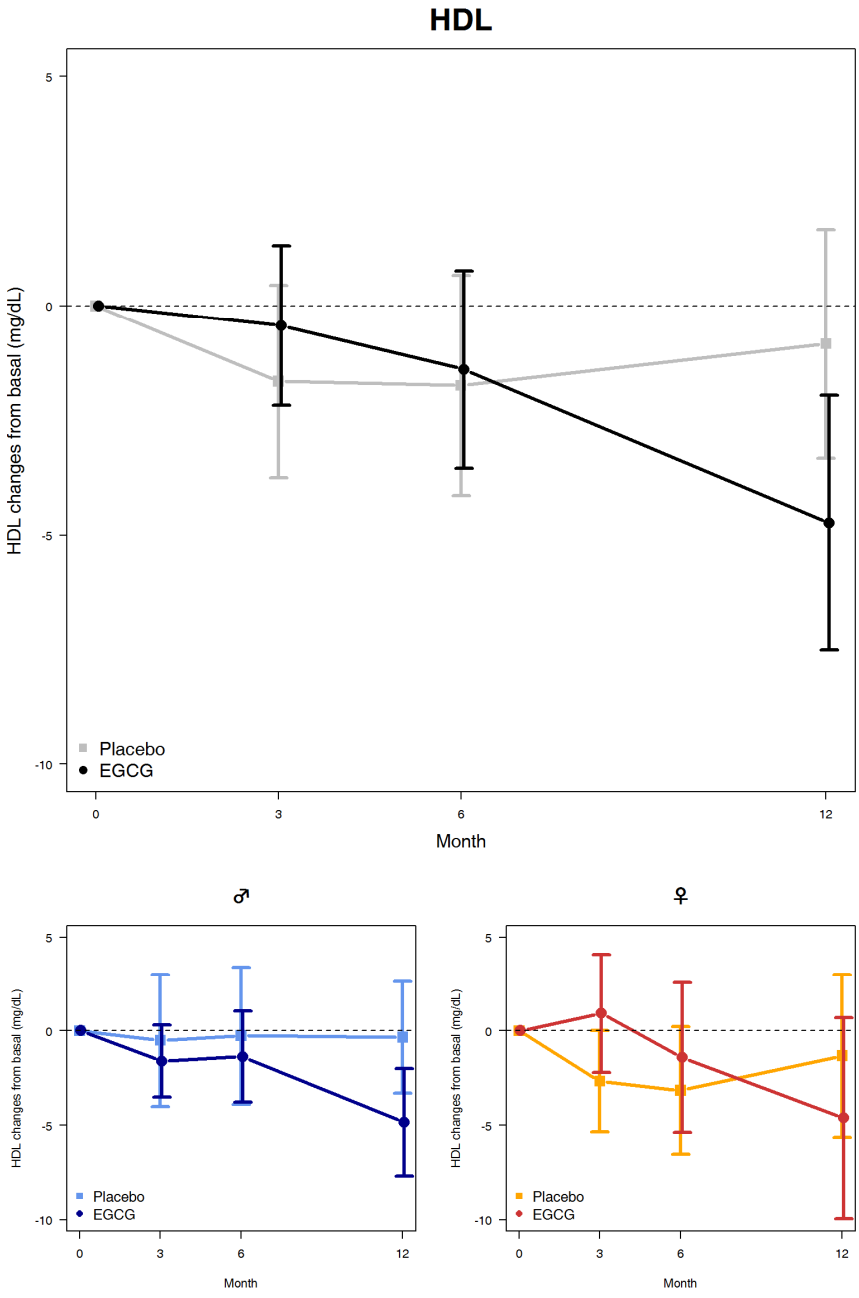

Figure 8: Changes in HDLc concentrations from baseline. On the top graph changes for the Placebo group are in light grey, while EGCG is in black. On the left bottom panel, stratified changes for males show differences between the EGCG group (dark blue) and the Placebo group (light blue). On the right bottom panel no differences are observed in females between Placebo group (light orange) and EGCG group (dark orange).

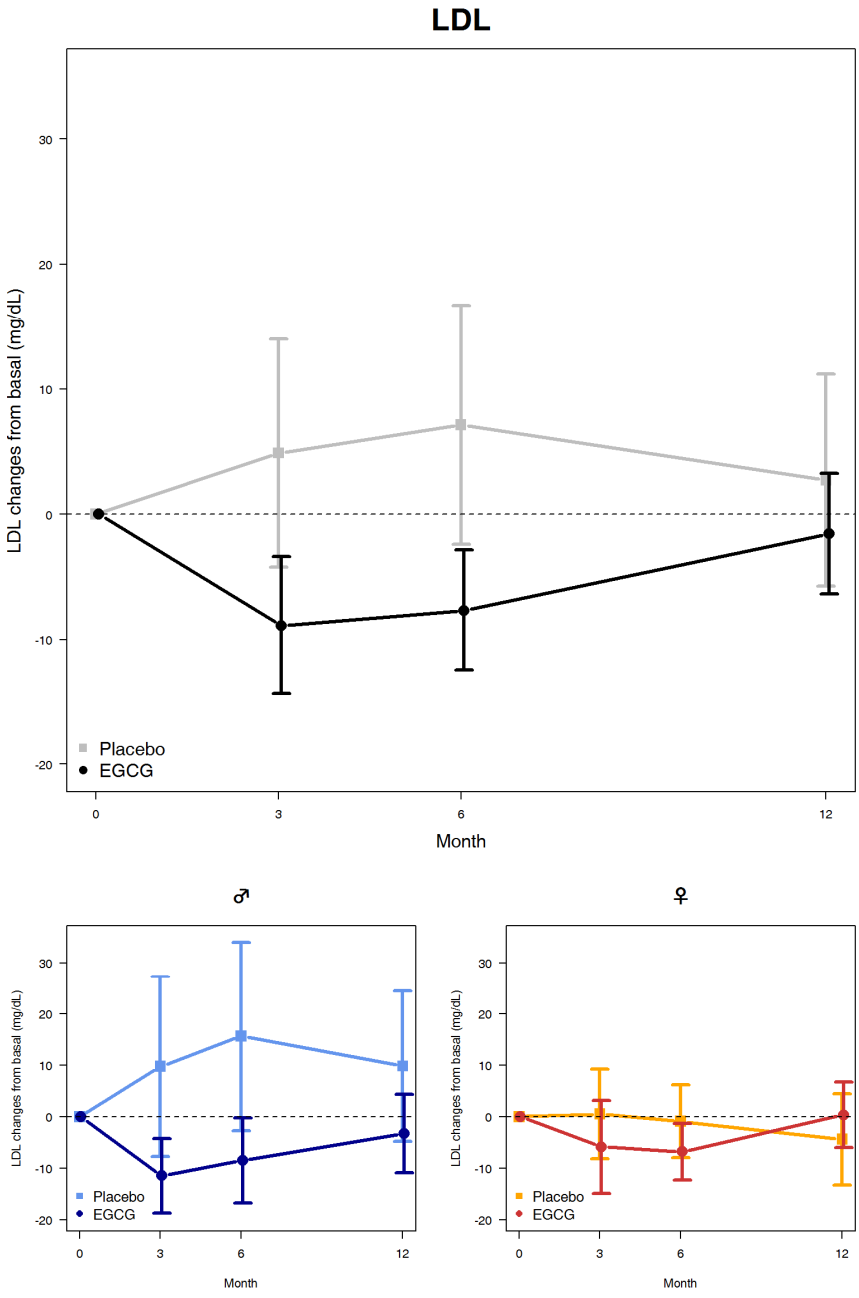

Figure 9: Changes in LDLc concentrations from baseline. On the top graph differences between the Placebo group (light grey) and the EGCG group (black) are observed. On the left bottom panel, stratified changes for males show differences between the EGCG group (dark blue) and the Placebo group (light blue). On the right bottom panel no differences are observed in females between Placebo group (light orange) and EGCG group (dark orange).

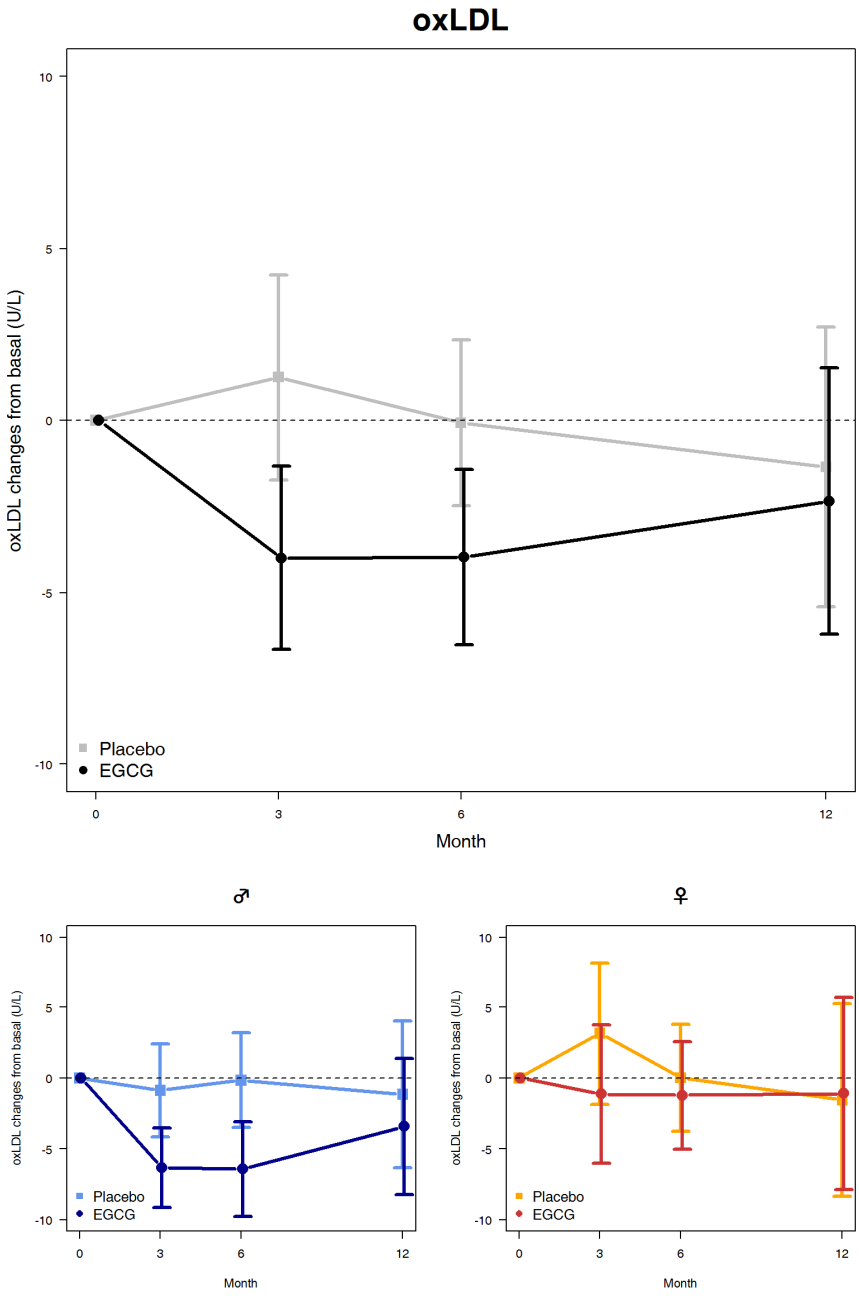

Figure 10: Changes in oxLDL concentrations from baseline. On the top graph differences between the Placebo group (light grey) and the EGCG group (black) are observed. On the left bottom panel, stratified changes for males show differences between the EGCG group (dark blue) and the Placebo group (light blue). On the right bottom panel no differences are observed in females between Placebo group (light orange) and EGCG group (dark orange).

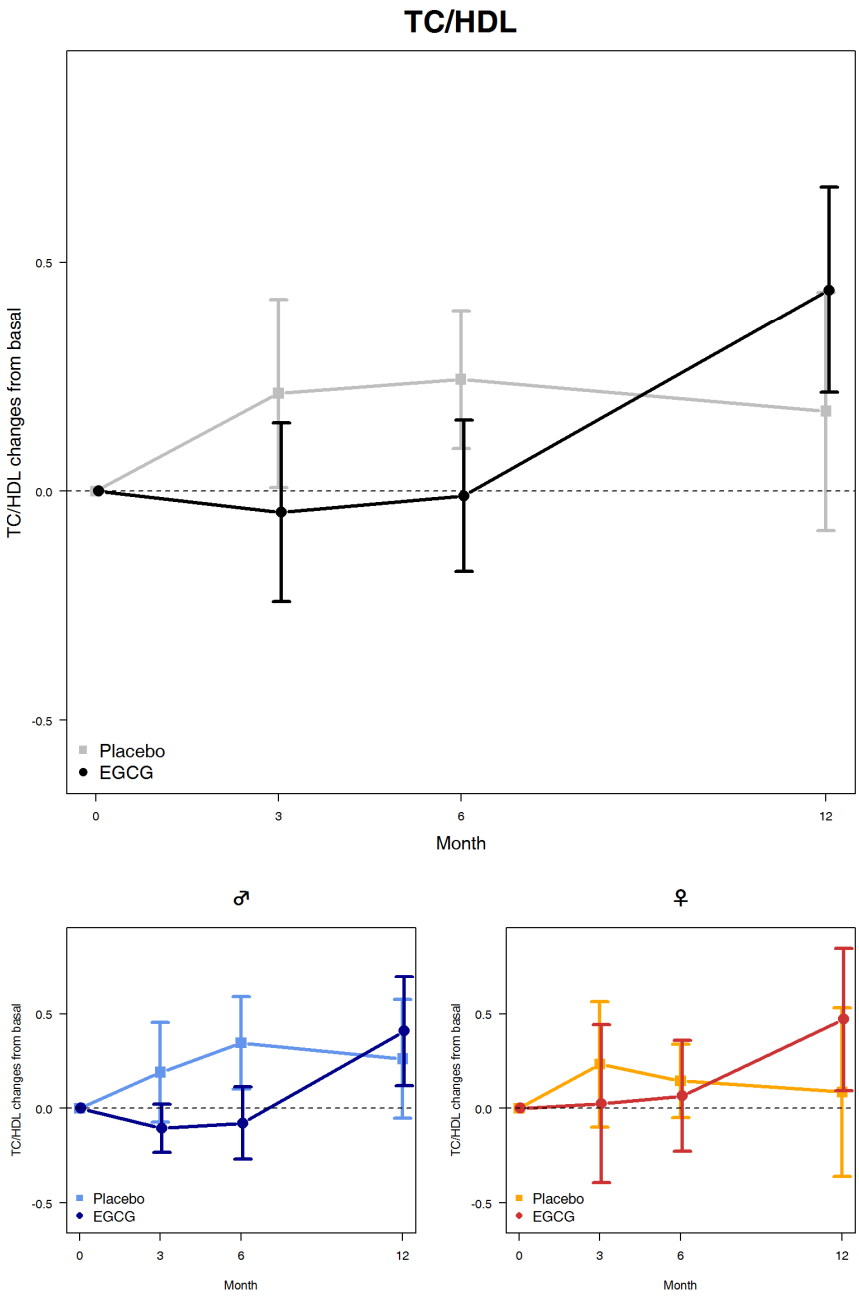

Figure 11: Changes in TC/HDLc from baseline. On the top graph differences between the Placebo group (light grey) and the EGCG group (black) are observed. On the left bottom panel, stratified changes for males show differences between the EGCG group (dark blue) and the Placebo group (light blue). On the right bottom panel no differences are observed in females between Placebo group (light orange) and EGCG group (dark orange).

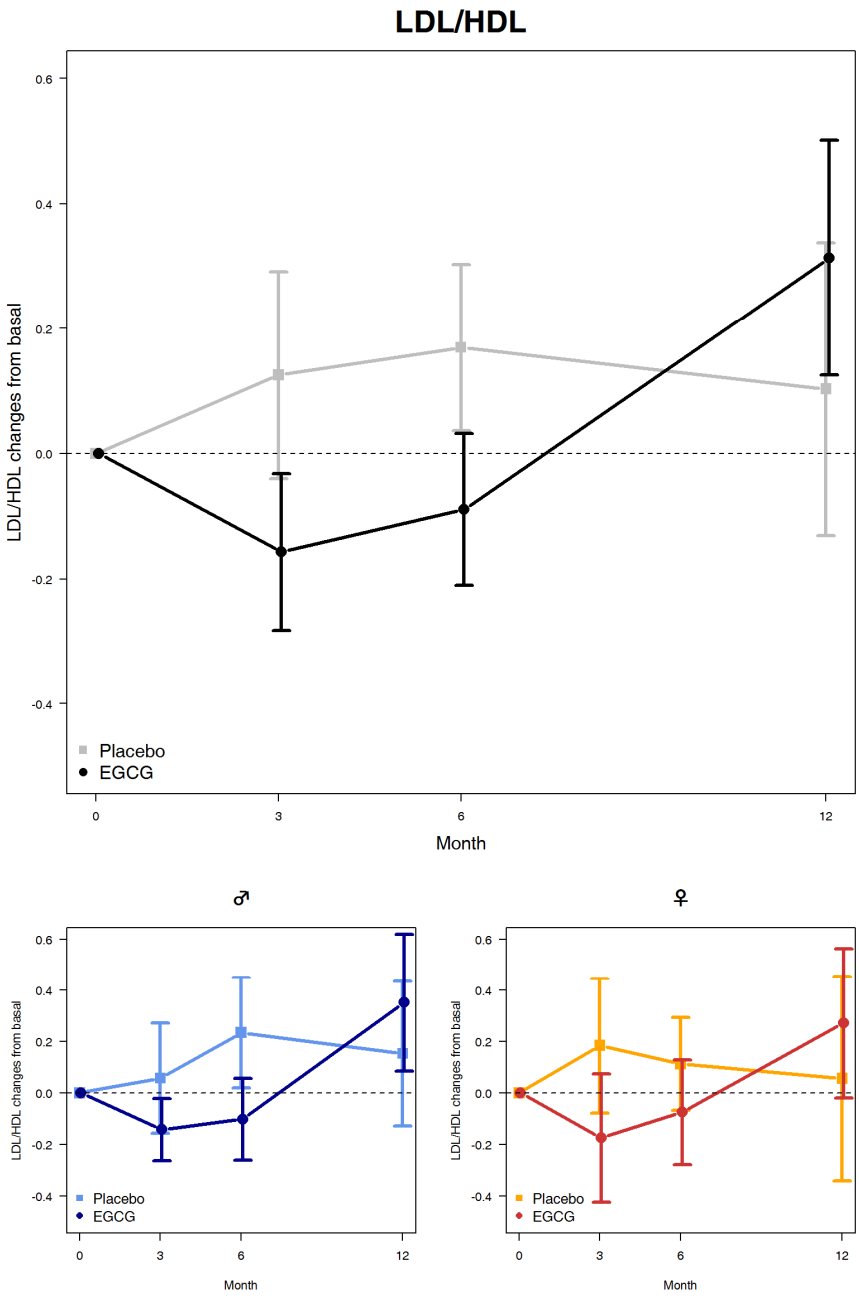

Figure 12: Changes in LDLc/HDLc from baseline. On the top graph differences between the Placebo group (light grey) and the EGCG group (black) are observed. On the left bottom panel, stratified changes for males show differences between the EGCG group (dark blue) and the Placebo group (light blue). The right bottom panel shows differences in females between Placebo group (light orange) and EGCG group (dark orange).

3.3.6. Effects of EGCG on the cognitive variables

From baseline associations between lipid profile components and cognitive performance, the following variables were selected for longitudinal studies: SSP span length, SSP backward span length, ToLDx solving time, correct and total movements, and from ABAS-II Functional Academics and Self-care subscales. Only the functional academics scale of ABASII was associated with EGCG+cognitive stimulation effects seen in the Phase II clinical trial (13). As with the lipid profile, new analyses for the study sample of the present Study 1 (n=77) were performed **(Supplementary Table 3)**, confirming the results from the trial as EGCG+cognitive stimulation treatment increased the ABAS-II Functional Academics subscale score at all time points and for both sexes in our and the whole sample (n=84) **(Figure 13)**.

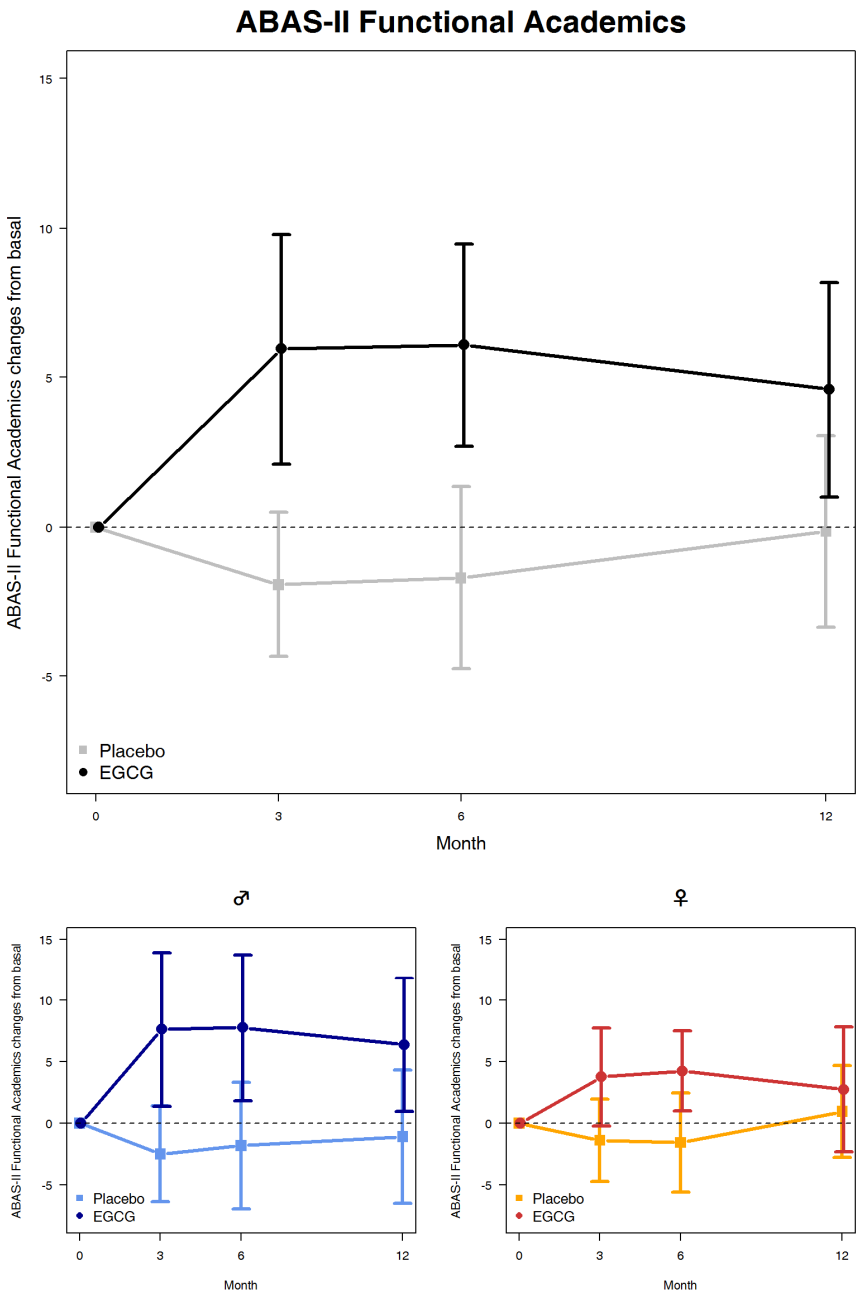

Figure 13: Changes in ABAS-II Functional Academics subscale from baseline. On the top graph differences between the Placebo group (light grey) and the EGCG group (black) are observed. On the left bottom panel, stratified changes for males show differences between the EGCG group (dark blue) and the Placebo group (light blue). The right bottom panel shows differences in females between Placebo group (light orange) and EGCG group (dark orange).

3.3.7. Longitudinal associations between changes in lipid profile and changes in cognition.

Models were performed to study the association between the changes in lipid profile and the changes in cognition along the whole study. Changes were understood as differences from one time-point to the previous one, and, thus, differences were calculated between 0 and 3 months, between 3 and 6 months, and between 6 and 12 months. General and sex-stratified analyses were performed for each of the treatment branches. Only those cognitive variables that had shown a significant association with components of the lipid profile were included. Full results can be found on **Supplementary Table 4.** The observed associations differed between treatment and sex groups. Significant associations were found between increases in LDL concentrations and increases in SSP Span Length in the EGCG group, a stratified analysis proved that a trend could be found in females of this group, but no association was found in males.

ToLDx was one of the variables in which changes in lipid profile were more strongly associated to changes in cognition. In this there was an association between an improvement in the total number of correct movements and increases in TC, oxLDL, and their respective rations and decreases in log(Trig), HDLc, LDLc, and the LDLc/HDLc and log(Trig/HDLc) ratios in females of the placebo group. For this cognitive variable no significant associations could be observed in the females of the EGCG group, although there was a trend for increases in log(Trig) and its ratio to be associated to a higher number of correct numbers in the unstratified EGCG group. Some of these associations

could also be observed in the ToLDx total movements variables, with females that increased the number of movements having decreases in both TC and its ratio, and increases in the log(Trig), LDLc, and their respective ratios. For the EGCG group only a significant association was found in the unstratified group between decreases in log(Trig/HDLc) and increases in the number of total movements. No associations regarding the ToLDx solving time could be observed in the placebo group. In the treated group, decreases in the log(Trig/HDLc) were associated with increases in the solving time, and this association was a trend for females of this same group.

For the functional variables, the associations were only observed in the ABAS-II Functional Academics subscale, the only studied variable in which there were differences between treatments. In this case in the placebo group a significant association between increases in oxLDL and its ratio, and improvements in this subscale have been observed. This association is significant for both the unstratified group and females, but not for males. In the EGCG group, such association disappears, however decreases in both log(Trig) and LDLc are associated to improvements in the functional variable in males.

3.4. **Discussion**

In this study we describe the gender dependent association between HDLc and specific components of the executive functions. In males this association was specific for spatial attention and working memory measures, while in females we detected it in planning skills. Regardless, there was an association with functionality which is of particular interest since it hints to a real impact on daily living. We also describe

the effect of changes in lipid profile have in cognition and how EGCG alters these associations.

3.4.1. Characteristics of the lipid profile in DS

One of the main modifiers of lipid profile is gender. Females are known to have higher HDLc and lower LDLc concentrations than males (89, 95). In our population we only observe differences in HDLc concentrations, but not in LDLc, therefore the DS phenotype could be affecting some of the effects of sex, among many other factors, has in lipid profile.

Likewise, ApoE genotype has also been described as having an impact on lipid concentrations. ε4 allele is associated to higher cholesterol concentrations and worse lipid profile, with ε2 having the opposite effect (89, 90). In our population the only observable differences were between ε2 carriers and ε3 homozygotes, however both sample size and distribution do not allow us to fully understand the effect of ApoE in this population.

Both TC and triglycerides concentrations have been described to increase with age in general population (89) and a decline of cholesterol concentrations has also been reported in the elderly. However, we observe the inverse association in our population, with older individuals having lower total and relative concentrations of Triglycerides. Possibly, this observed association is due to our limited age-range and a study with a wider age-range would be needed to confirm this observation.

3.4.2. Effects of gender, age, and ApoE in EF

Regarding cognition, females had higher K-BIT scores and better planning skills as measured through ToLDx. However, no other differences between genders were observed. This lack of differences is particularly interesting when it comes to the functional variables, as it shows that the described differences do not translate into differences in everyday functionality.

ApoE genotype has previously been described to have an association with cognition in general population. Specifically, ε4 and ε2 female carriers had higher IQs than ε3 homozygotes (105), and with SVFT where ε4 carriers had a better performance than ε2 carriers (152). We do not observe any effect of ApoE on IQ or K-BIT scores, although this could be due to sample size. On the other hand, we do observe the contrary effect on SVFT, with ε2 carriers performing better, further research will be needed to ascertain this effect. This effect will also be explored in **Chapter III**.

The fact that no effect of age could be observed is probably due to the age distribution of our population, as cognitive decline has not yet started.

3.4.3. Effects of MeDi compliance

The MeDi pattern has long been associated to a lower risk of AD (153) and cognitive decline (154, 155) in general population. This beneficial effect has also been observed in interventional studies, with individuals receiving a MeDi pattern plus extra virgin olive oil had better cognition and lower MCI (124, 156). Interestingly, the possible

protective effects of MeDi are not restricted to AD risk as higher adherence to MeDi has been associated to better performance in specific cognitive domains such as episodic memory as well as on MMSE scores (155). No previous study has analyzed the effects of MeDi on either lipid profile or cognition in DS. We could not observe an effect of MeDi compliance on lipid profile, but there was a clear association between MeDi compliance and functionality measures, especially with the Self-care and Total scores of the ABAS-II. There is also an association between MeDi compliance and lower scores in the DMR questionnaire. The only cognitive task in which there was an association with MeDi compliance was the Direct Digit Span, with individuals in the high compliance group having higher verbal attention spans. The combination of these results hints to a potential beneficial effect of MeDi on functionality and cognitive decline in population with DS. However, more research with a larger number of individuals would be needed to ascertain this claim.

3.4.4. Sex-dependent associations between HDLc and executive functions

Our results show a sex-dependent association between HDLc and executive functions. These different associations could well be related to the differences in both lipid profile and cognition, with females having higher concentrations of HDLc and better performance in ToLDx. Incidentally, the associations between HDLc and ToLDx are only seen in females, associating higher concentrations of HDLc to better planning skills. In males, the only significant observable association with HDLc was with visuo-spatial attention, which was

not different between males and females. Independently of gender, higher HDLc concentrations were associated to higher ABAS-II Functional Academics subscale scores, implying that higher concentrations of HDLc are beneficial regardless of gender.

Cholesterol also presented sex-dependent associations with the same cognitive variables. In this case, higher cholesterol was associated with worse planning skills in females and worse spatial attention in males. Independently of HDLc, there was a direct association between cholesterol and ABAS-II Self-care subscale. For this particular subscale there is a MeDi compliance effect that could be affecting the association between the functional variable and cholesterol. However, no effect of MeDi was observed in TC concentrations, thus more research will be needed to explore this possible association.

Both triglycerides and LDLc were only associated with planning skills in females, with higher concentrations being related to better performance.

These associations show how the lipid profile has a strong influence on executive functions, in particular on planning and attention, with a specific interest on HDLc as its concentrations are also associated with functionality.

3.4.5. oxLDL and cognition, an unexplored association

Of the HDLc-independent associations the most unexpected was the direct association between both total and relative oxLDL concentrations and better spatial working memory in males, as well as

with the ABAS-II Functional Academics subscale. Such association has never been described before, neither in the general population nor in DS, even though it is generally accepted that higher concentrations of oxLDL reflect a higher oxidative status that is detrimental for cognition (157, 158). In order to better understand this association and, taking into account both the prevalence of thyroid dysfunction in population with DS (108) and the association of both hypo and hyperthyroidism with higher oxidative stress (107), we studied the effect of thyroid hormones, other components of the lipid profile, gender, and age on the concentrations of oxLDL. Our results show that the only significant association is between fT_4 and oxLDL, with higher concentrations of the thyroid hormone being associated to higher concentrations of the peripheral lipid (Estimate=9.774, SE=4.124, p=0.022). This association between oxLDL and fT_4 could explain the one between oxLDL and working memory performance, although no effect of fT_4 on the cognitive variables could be observed when it was introduced in the model.

3.4.6. Effect of EGCG on the Lipid profile

As described in de la Torre *et al* 2016 (13) **(Annex 1)**, the concentration changes from baseline were significantly different between treatments for TC and LDLc, however no differences could be observed for HDLc. Additionally, our results also show differences in intermediate time points for the TC/HDLc and LDLc/HDLc ratios, with the ratios decreasing in the EGCG group. Additionally, the stratified analysis shows differences according to sex for the effects of EGCG on lipid profile. In this case, the differences in total concentrations between treatments are only significant in males, with

those in the EGCG treatment having higher decreases in TC, LDLc, HDLc, and oxLDL concentrations, and lower increases in Triglycerides concentrations. The stratified analysis for the ratios also shows differences for the associations TC/HDLc in males.

Therefore, our results show a sex-specific pattern for the effects of EGCG on the lipid profile, with males being more sensitive to the effects of EGCG. This sex-specificity is described here for the first time but further research will be needed to assess whether it is also present in the general population.

3.4.7. Effect of EGCG on executive functions

In the TESDAD clinical trial significant treatment differences could be observed at 12 months for the Paired Recognition Memory (PRM) immediate recall, the Cats and Dogs total time and correct score, and the ABAS-II Functional Academics subscale (13) **(See Annex 1)**. In our subpopulation, only the ABAS-II Functional Academics subscale was included in the analysis. Our results show the same effect of the treatment combination of EGCG and cognitive training, and the stratified results do not show any differences on the effects between genders.

All the other variables included in our analysis showed improvements regardless of treatment or sex.

3.4.8. Longitudinal associations between changes in lipid profile and changes in cognition

We observed associations with the lipid profile in three main domains, visuo-spatial attention, planning skills, and functionality.

The association between SSP Span Length and LDLc concentrations had already been observed in basal conditions in males. In the longitudinal analysis the changes in the cognitive variable are associated with LDLc in both the EGCG group and in females treated with EGCG, with the results pointing more to a specific effect in females. It is worth noting that in females neither the changes in cognition nor in LDLc are significantly different between treatment groups. Furthermore, although non-significant, the association between changes in SSP Span Length and changes in LDLc in females in the placebo group are in the same direction than those of the EGCG group.

Associations between ToLDx performance and lipid concentrations had been already observed in females in basal conditions. The fact that most of our observed associations are in the females in the placebo group would imply that the lipid profile concentrations could have a real association with the changes in the performance in this task. In this case the effects observed in females of the EGCG group are non-significant, of a much smaller magnitude, and, in some cases, opposite to those seen in the placebo group.

The association between higher oxLDL concentrations and higher scores in the ABAS-II Functional Academics subscale was already

observed in basal conditions and in the longitudinal analysis still seems to be associated to increases of those in the placebo group, albeit mainly in females. This association would reinforce the idea that there is some kind of compensatory mechanism that enhances functionality at the cost of increased oxidation. EGCG would break this circle, reducing oxLDL concentrations while at the same time increasing the scores in ABAS-II: Functional Academics subscale. Furthermore, specific associations in the EGCG group between changes in lipid profile and changes in this functional variable were observed. In this case decreases in triglycerides and LDLc concentrations were associated to increases in the scores, which could shed light to one of the possible mechanisms of EGCG.

3.5. **Limitations**

This study has several limitations. On one hand, due to sample size, we could not fully measure the potential effects of ApoE genotype on both cognition and lipid profile. Additionally, questionnaires of diet compliance could not be obtained for all the participants (60 over 77), and thus we could not fully assess the influence of MeDi compliance on our variables. The nature of the design, a transversal one, does not allow us to fully establish causality. Finally, more oxidation markers should have been obtained in order to better understand the association between oxLDL and working memory and functionality.

Our sample size and the posterior stratifications did not allow us to perform corrections for multiple comparisons. Additionally, in the longitudinal analysis we could not include the genotype and MeDi compliance on the analysis, as it would reduce the sample size.

In light of our results a longitudinal study assessing the effect of lipid concentrations on cognitive function in DS at all time-points during lifetime is needed. Likewise, a full analysis of the effects of MeDi on dementia risk and cognition could shed light to the potential protective risk this dietary pattern could have.

3.6. **Conclusions**

Lipid profile, and in particular HDLc, is associated with specific components of executive functions, and functional variables, in young individuals with DS. Taking into account that this population has decreased concentrations of HDLc, when compared to control population, and that executive functions are severely impaired in individuals with DS, this association is of great importance. We have also observed a potential beneficial effect of MeDi in this population, with those in the higher compliance group having better functionality and lower signs of cognitive decline.

The observed associations are of potential interest in this population with cognitive disabilities since an intervention on lipid profile could have an impact on their executive functions.

EGCG has a sex-dependent effect on the lipid profile, although not in cognition. The longitudinal associations between lipid profile and cognition were also dependent on sex and treatment, with some of the associations observed in the Placebo group being cancelled in the EGCG one, and specific associations for the EGCG group in the only variable in which there are differences between treatments. These associations suggest that one of the mechanisms through which

EGCG would be exerting its effect could be the modification of the lipid profile.

In conclusion, lipid profile has shown its potential as biomarker of cognition and cognitive change, however when using it as such it is crucial to take into account its sex-dependent differences.

3.7. **Supplementary tables**

Supplementary Table 1: Basal associations between lipid profile components and cognition.

			Total	Males	Females
K-BIT	TC	Estimate	-0.278	0.728	-2.206
		SE	1.238	1.675	1.798
		p	0.82	0.67	0.23
	log(Trig)	Estimate	11.739	-9.015	*41.856*
		SE	17.147	23.985	*23.806*
		p	0.5	0.71	*0.09*
	HDLc	Estimate	0.493	-1.121	2.391
		SE	1.223	1.667	1.760
		p	0.69	0.51	0.19
	LDLc	Estimate	0.023	-0.809	1.891
		SE	1.262	1.713	1.829
		p	0.99	0.64	0.31
	oxLDL	Estimate	0.142	0.127	-0.043
		SE	0.184	0.318	0.231
		p	0.44	0.69	0.85
	TC/HDLc	Estimate	4.246	6.373	-13.40
		SE	40.314	55.864	61.75
		p	0.916	0.91	0.83
	log(Trig/HDLc)	Estimate	6.103	1.703	15.97
		SE	13.055	19.050	18.28
		p	0.64	0.93	0.39
	LDLc/HDLc	Estimate	-12.129	-7.331	1.73
		SE	41.234	57.118	63.86
		p	0.77	0.9	0.98
	oxLDLc/LDLc	Estimate	28.277	28.827	14.64
		SE	19.509	34.066	25.55
		p	0.15	0.4	0.57
SSP Span Length	TC	Estimate	*-0.207*	**-0.379**	-0.027
		SE	*0.113*	**0.182**	0.116
		p	*0.07*	**0.045**	0.82
	log(Trig)	Estimate	2.626	4.860	0.471
		SE	1.581	2.638	1.541
		p	0.1	*0.075*	0.76
	HDLc	Estimate	**0.239**	**0.370**	0.054
		SE	**0.112**	**0.181**	0.114
		p	**0.036**	**0.049**	0.64

			Total	Males	Females
	LDLc	Estimate	*0.202*	**0.378**	0.017
		SE	*0.115*	**0.185**	0.118
		p	*0.08*	**0.05**	0.89
	oxLDL	Estimate	*0.029*	0.058	0.006
		SE	*0.017*	0.035	0.015
		p	*0.097*	0.11	0.71
	TC/HDLc	Estimate	-4.318	*-11.927*	3.356
		SE	3.795	*6.092*	3.801
		p	0.26	*0.059*	0.38
	log(Trig/HDLc)	Estimate	0.959	3.454	-0.787
		SE	1.245	2.112	1.125
		p	0.44	0.11	0.49
	LDLc/HDLc	Estimate	4.328	*12.468*	-3.874
		SE	3.875	*6.212*	3.931
		p	0.27	*0.053*	0.33
	oxLDL/LDLc	Estimate	2.494	*6.624*	0.059
		SE	1.867	*3.743*	1.573
		p	0.19	*0.086*	0.97
SSP Backward Span Length	TC	Estimate	-0.019	0.121	-0.298
		SE	0.113	0.134	0.189
		p	0.86	0.37	0.13
	log(Trig)	Estimate	0.594	-1.593	*4.528*
		SE	1.584	1.943	*2.507*
		p	0.71	0.42	*0.081*
	HDLc	Estimate	0.059	-0.080	0.307
		SE	0.112	0.133	0.185
		p	0.59	0.55	0.11
	LDLc	Estimate	-0.006	-0.143	0.271
		SE	0.115	0.137	0.192
		p	0.96	0.3	0.17
	oxLDL	Estimate	**0.042**	**0.067**	0.005
		SE	**0.017**	**0.026**	0.024
		p	**0.016**	**0.014**	0.83
	TC/HDLc	Estimate	1.328	3.633	-3.031
		SE	3.658	4.555	6.369
		p	0.72	0.43	0.64
	log(Trig/HDLc)	Estimate	-0.266	-1.227	1.386
		SE	1.200	1.579	1.884
		p	0.825	0.44	0.47
	LDLc/HDLc	Estimate	-1.867	-3.705	2.235
		SE	3.736	4.645	6.586
		p	0.62	0.43	0.74

			Total	Males	Females
	oxLDL/LDLc	Estimate	**5.010**	**6.916**	2.943
		SE	**1.853**	**2.799**	2.727
		p	**0.009**	**0.019**	0.29
Direct Digit Span	TC	Estimate	-0.034	0.011	-0.106
		SE	0.063	0.099	0.085
		p	0.59	0.91	0.22
	log(Trig)	Estimate	0.269	-0.412	1.066
		SE	0.883	1.431	1.127
		p	0.76	0.78	0.35
	HDLc	Estimate	0.035	-0.022	0.104
		SE	0.062	0.098	0.083
		p	0.57	0.82	0.22
	LDLc	Estimate	0.033	-0.013	0.106
		SE	0.064	0.101	0.087
		p	0.21	0.9	0.23
	oxLDL	Estimate	0.006	0.005	0.007
		SE	0.010	0.019	0.011
		p	0.55	0.79	0.55
	TC/HDLc	Estimate	0.059	-0.213	-0.783
		SE	2.046	3.249	2.757
		p	0.98	0.95	0.78
	log(Trig/HDLc)	Estimate	-0.276	-0.076	-0.283
		SE	0.671	1.126	0.816
		p	0.68	0.95	0.73
	LDLc/HDLc	Estimate	0.010	0.204	1.034
		SE	2.089	3.313	2.851
		p	0.99	0.95	0.72
	oxLDL/LDLc	Estimate	1.349	1.632	1.575
		SE	1.006	1.997	1.141
		p	0.18	0.42	0.18
Inverse Digit Span	TC	Estimate	-0.106	-0.163	-0.002
		SE	0.090	0.122	0.150
		p	0.24	0.19	0.99
	log(Trig)	Estimate	1.346	1.864	0.384
		SE	1.255	1.769	1.980
		p	0.29	0.29	0.85
	HDLc	Estimate	0.137	0.182	0.033
		SE	0.089	0.121	0.146
		p	0.13	0.14	0.82
	LDLc	Estimate	0.102	0.158	-0.003
		SE	0.091	0.124	0.152
		p	0.27	0.21	0.99

			Total	Males	Females
	oxLDL	Estimate	**0.029**	*0.041*	0.021
		SE	**0.014**	*0.023*	0.019
		p	**0.034**	*0.088*	0.27
	TC/HDLc	Estimate	-2.309	-3.626	-1.033
		SE	3.051	4.122	5.049
		p	0.45	0.38	0.84
	log(Trig/HDLc)	Estimate	0.380	0.679	0.429
		SE	1.001	1.429	1.494
		p	0.71	0.64	0.78
	LDLc/HDLc	Estimate	2.414	3.871	0.933
		SE	3.116	4.203	5.221
		p	0.44	0.36	0.86
	oxLDL/LDLc	Estimate	2.165	*4.722*	0.363
		SE	1.501	*2.533*	2.089
		p	0.15	*0.071*	0.86
Total Animals	TC	Estimate	-0.361	-0.573	-0.204
		SE	0.339	0.500	0.517
		p	0.29	0.26	0.7
	LDLc	Estimate	0.342	0.569	0.171
		SE	0.345	0.509	0.526
		p	0.33	0.27	0.75
	oxLDLc	Estimate	0.054	0.077	0.018
		SE	0.051	0.096	0.066
		p	0.3	0.43	0.78
	HDLc	Estimate	0.412	0.598	0.244
		SE	0.335	0.498	0.506
		p	0.22	0.24	0.63
	log(Trig)	Estimate	4.246	7.309	2.323
		SE	4.743	7.250	6.841
		p	0.37	0.32	0.74
	TC/HDLc	Estimate	-11.325	-19.154	-4.382
		SE	10.988	16.420	16.622
		p	0.31	0.25	0.79
	log(Trig/HDLc)	Estimate	2.778	5.582	1.071
		SE	3.606	5.692	4.920
		p	0.44	0.33	0.83
	LDLc/HDLc	Estimate	10.916	19.393	3.331
		SE	11.221	16.744	17.190
		p	0.33	0.26	0.85
	oxLDL/LDLc	Estimate	5.611	11.471	2.551
		SE	5.405	10.089	6.879
		p	0.3	0.26	0.71

			Total	Males	Females
Cats and dogs inhibition score	TC	Estimate	0.059	0.226	-0.077
		SE	0.125	0.208	0.159
		p	0.64	0.29	0.63
	log(Trig)	Estimate	-0.399	-2.668	1.462
		SE	1.636	2.708	2.102
		p	0.81	0.33	0.49
	HDLc	Estimate	-0.048	-0.247	0.094
		SE	0.122	0.203	0.155
		p	0.7	0.23	0.55
	LDLc	Estimate	-0.033	-0.246	0.075
		SE	0.126	0.210	0.162
		p	0.6	0.25	0.65
	oxLDL	Estimate	-0.001	0.027	-0.013
		SE	0.016	0.028	0.020
		p	0.95	0.34	0.53
	TC/HDLc	Estimate	0.471	7.903	-3.383
		SE	4.067	7.374	5.082
		p	0.91	0.29	0.51
	log(Trig/HDLc)	Estimate	0.259	-1.924	1.504
		SE	1.232	2.252	1.504
		p	0.83	0.4	0.32
	LDLc/HDLc	Estimate	-0.844	-8.166	2.948
		SE	4.156	7.475	5.256
		p	0.84	0.28	0.58
	oxLDL/LDLc	Estimate	-0.472	3.477	-2.224
		SE	1.675	3.291	2.103
		p	0.78	0.3	0.3
Cats and dogs inhibition time	TC	Estimate	0.753	-1.405	2.487
		SE	1.279	2.106	1.544
		p	0.56	0.51	0.12
	log(Trig)	Estimate	-12.375	21.324	*-41.646*
		SE	16.802	27.410	*20.445*
		p	0.46	0.44	*0.0503*
	LDLc	Estimate	-0.645	1.644	-2.424
		SE	1.299	2.129	1.571
		p	0.62	0.45	0.13
	oxLDL	Estimate	-0.019	-0.43	0.222
		SE	0.161	0.287	0.198
		p	0.9	0.15	0.27
	HDLc	Estimate	-0.835	1.792	*-2.621*
		SE	1.255	2.056	*1.512*
		p	0.5	0.39	*0.093*

			Total	Males	Females
	TC/HDLc	Estimate	23.783	-48.27	55.10
		SE	41.730	75.55	50.49
		p	0.57	0.53	0.28
	log(Trig/HDLc)	Estimate	-9.811	14.78	*-26.18*
		SE	12.641	23.07	*14.94*
		p	0.44	0.53	*0.09*
	LDLc/HDLc	Estimate	-19.889	49.08	-47.83
		SE	42.635	76.59	52.21
		p	0.64	0.53	0.37
	oxLDL/LDLc	Estimate	-5.725	*-58.16*	24.88
		SE	17.183	*33.72*	20.89
		p	0.74	*0.096*	0.24
ToLDx resolution time	TC	Estimate	30.481	3.070	*54.696*
		SE	19.952	31.016	*27.017*
		p	0.13	0.92	*0.052*
	log(Trig)	Estimate	-100.959	8.804	**-731.417**
		SE	276.546	446.686	**349.023**
		p	0.15	0.98	**0.045**
	HDLc	Estimate	*-37.476*	-15.458	**-59.984**
		SE	*19.539*	30.437	**26.363**
		p	*0.06*	0.62	**0.03**
	LDLc	Estimate	-26.425	2.901	*-51.890*
		SE	20.291	31.657	*27.446*
		p	0.2	0.93	*0.068*
	oxLDL	Estimate	-4.057	-4.609	-4.772
		SE	3.059	6.131	3.378
		p	0.19	0.46	0.17
	TC/HDLc	Estimate	437.33	-238.3	1153.0
		SE	658.59	1028.4	884.8
		p	0.51	0.82	0.2
	log(Trig/HDLc)	Estimate	-124.67	119.0	-338.4
		SE	217.63	356.6	265.2
		p	0.57	0.74	0.21
	LDLc/HDLc	Estimate	-311.79	440.6	-1088.2
		SE	672.47	1050.1	915.2
		p	0.64	0.68	0.24
	oxLDL/LDLc	Estimate	-410.63	-468.7	-381.0
		SE	332.13	658.1	387.0
		p	0.22	0.48	0.33
ToLDx Total Correct Movements	TC	Estimate	-0.159	-0.029	**-0.368**
		SE	0.107	0.146	**0.162**
		p	0.14	0.85	**0.03**

			Total	Males	Females
	log(Trig)	Estimate	2.045	-0.016	**4.631**
		SE	1.485	2.104	**2.088**
		p	0.17	0.99	**0.034**
	HDLc	Estimate	0.197	0.033	**0.395**
		SE	0.105	0.143	**0.158**
		p	*0.067*	0.82	**0.018**
	LDLc	Estimate	0.141	0.027	**0.349**
		SE	0.109	0.149	**0.164**
		p	0.19	0.86	**0.042**
	oxLDL	Estimate	0.024	0.008	0.024
		SE	0.016	0.029	0.020
		p	0.14	0.8	0.25
	TC/HDLc	Estimate	-3.573	0.029	**-10.258**
		SE	3.449	4.786	**5.009**
		p	0.3	0.99	**0.049**
	log(Trig/HDLc)	Estimate	0.933	-0.361	*2.835*
		SE	1.140	1.660	*1.502*
		p	0.42	0.83	*0.068*
	LDLc/HDLc	Estimate	3.082	-0.108	*9.830*
		SE	3.521	4.887	*5.182*
		p	0.38	0.98	*0.067*
	oxLDL/LDLc	Estimate	3.019	2.423	2.407
		SE	1.739	3.063	2.191
		p	0.087	0.44	0.28
ToLDx Total Movements	TC	Estimate	3.236	0.014	5.963
		SE	2.867	4.398	3.884
		p	0.26	0.99	0.14
	log(Trig)	Estimate	-50.419	7.037	*-93.201*
		SE	39.734	63.337	*50.173*
		p	0.21	0.91	*0.073*
	HDLc	Estimate	-4.579	-0.553	-7.475
		SE	2.807	4.316	3.790
		p	0.11	0.9	*0.058*
	LDLc	Estimate	-2.799	0.459	-5.632
		SE	2.915	4.489	3.946
		p	0.34	0.92	0.16
	oxLDL	Estimate	-0.183	0.135	-0.463
		SE	0.440	0.869	0.486
		p	0.68	0.88	0.35
	TC/HDLc	Estimate	38.882	-43.322	134.40
		SE	95.807	146.112	135.22
		p	0.7	0.77	0.33

			Total	Males	Females
	log(Trig/HDLc)	Estimate	-16.454	16.129	-50.93
		SE	31.659	50.669	40.54
		p	0.6	0.75	0.22
	LDLc/HDLc	Estimate	-18.573	66.121	-117.24
		SE	97.827	149.207	139.88
		p	0.85	0.66	0.41
	oxLDL/LDLc	Estimate	3.992	-4.732	25.31
		SE	48.316	93.499	59.15
		p	0.93	0.96	0.67
ABAS-II	TC	Estimate	-0.168	1.403	*-2.105*
Community Use		SE	1.048	1.519	*1.547*
		p	0.87	0.36	*0.09*
	log(Trig)	Estimate	0.634	-0.755	*3.001*
		SE	1.034	1.512	*1.515*
		p	0.54	0.62	*0.057*
	HDLc	Estimate	-2.815	-23.546	27.641
		SE	14.649	22.033	20.487
		p	0.85	0.29	0.19
	LDLc	Estimate	0.105	-1.409	2.653
		SE	1.067	1.548	1.574
		p	0.92	0.37	0.1
	oxLDL	Estimate	0.208	0.118	0.156
		SE	0.158	0.291	0.199
		p	0.19	0.69	0.44
	TC/HDLc	Estimate	23.40	57.920	-34.614
		SE	34.71	51.405	52.494
		p	0.5	0.27	0.51
	log(Trig/HDLc)	Estimate	-13.98	-25.200	0.522
		SE	11.39	17.819	15.536
		p	0.22	0.17	0.97
	LDLc/HDLc	Estimate	-25.28	-58.651	34.341
		SE	35.45	52.418	54.286
		p	0.48	0.27	0.53
	oxLDL/LDLc	Estimate	15.56	9.691	16.978
		SE	17.08	31.586	21.723
		p	0.36	0.76	0.44
ABAS-II	TC	Estimate	0.748	**1.723**	-0.793
Self-care		SE	0.615	**0.806**	0.974
		p	0.23	**0.04**	0.42
	log(Trig)	Estimate	-9.854	*-22.455*	7.865
		SE	8.592	*11.687*	12.892
		p	0.26	*0.064*	0.55

			Total	Males	Females
	HDLc	Estimate	-0.373	-1.324	1.148
		SE	0.607	0.802	0.953
		p	0.54	0.11	0.24
	LDLc	Estimate	-0.781	*-1.649*	0.743
		SE	0.626	*0.821*	0.991
		p	0.22	*0.053*	0.46
	oxLDL	Estimate	0.088	-0.139	0.165
		SE	0.093	0.154	0.125
		p	0.35	0.37	0.2
	TC/HDLc	Estimate	*36.851*	**57.962**	4.337
		SE	*20.737*	**2.101**	33.665
		p	*0.08*	**0.043**	0.9
	log(Trig/HDLc)	Estimate	*-12.321*	*-19.372*	-5.043
		SE	*6.805*	*9.561*	9.964
		p	*0.075*	*0.051*	0.62
	LDLc/HDLc	Estimate	-39.391	**-58.503**	-6.331
		SE	21.177	**28.126**	34.814
		p	*0.067*	**0.045**	0.86
	oxLDL/LDLc	Estimate	-2.148	-24.004	7.519
		SE	10.201	16.948	13.931
		p	0.83	0.1	0.59
ABAS-II	TC	Estimate	0.016	0.869	-1.368
Self-direction		SE	1.170	1.613	1.872
		p	0.99	0.59	0.47
	log(Trig)	Estimate	-3.022	-14.743	15.092
		SE	16.357	23.388	24.790
		p	0.85	0.53	0.55
	HDLc	Estimate	0.472	-0.190	1.719
		SE	1.155	1.605	1.833
		p	0.68	0.9	0.36
	LDLc	Estimate	-0.044	-0.721	1.279
		SE	1.191	1.643	1.905
		p	0.97	0.66	0.51
	oxLDL	Estimate	0.124	-0.115	0.098
		SE	0.177	0.309	0.241
		p	0.48	0.71	0.69
	TC/HDLc	Estimate	38.039	45.265	34.487
		SE	38.722	55.351	61.632
		p	0.33	0.42	0.58
	log(Trig/HDLc)	Estimate	-16.208	-20.919	-12.980
		SE	12.706	19.187	18.241
		p	0.21	0.28	0.48

			Total	Males	Females
	LDLc/HDLc	Estimate	-40.487	-43.996	-38.947
		SE	39.543	56.443	63.736
		p	0.31	0.44	0.55
	oxLDL/LDLc	Estimate	0.778	-23.402	5.851
		SE	19.047	34.011	25.504
		p	0.97	0.49	0.82
ABAS-II Functional Academics	TC	Estimate	-2.111	-1.912	-2.754
		SE	1.299	1.915	1.884
		p	0.11	0.33	0.15
	log(Trig)	Estimate	17.784	13.953	25.336
		SE	18.166	27.772	24.945
		p	0.33	0.62	0.32
	HDLc	Estimate	**2.601**	2.313	3.034
		SE	**1.283**	1.906	1.845
		p	**0.046**	0.23	0.11
	LDLc	Estimate	2.019	1.823	2.671
		SE	1.323	1.951	1.917
		p	0.13	0.36	0.17
	oxLDL	Estimate	**0.435**	0.600	0.262
		SE	**0.196**	0.367	0.242
		p	**0.03**	0.11	0.29
	TC/HDLc	Estimate	-28.362	-48.631	-27.33
		SE	43.639	64.864	63.07
		p	0.52	0.46	0.67
	log(Trig/HDLc)	Estimate	-4.524	2.012	-4.54
		SE	14.319	22.485	18.67
		p	0.75	0.93	0.81
	LDLc/HDLc	Estimate	29.367	52.514	27.93
		SE	44.565	66.143	65.23
		p	0.51	0.43	0.67
	oxLDL/LDLc	Estimate	41.185	57.124	31.39
		SE	21.466	39.856	26.10
		p	0.059	0.16	0.24
Total ABAS-II	TC	Estimate	-1.082	5.689	-13.262
		SE	6.978	10.326	10.307
		p	0.88	0.59	0.21
	log(Trig)	Estimate	-12.543	-100.203	129.232
		SE	97.551	149.768	136.457
		p	0.9	0.51	0.35
	HDLc	Estimate	4.334	-2.114	15.685
		SE	6.887	10.278	10.091
		p	0.53	0.84	0.13

			Total	Males	Females
	LDLc	Estimate	0.686	-5.578	12.833
		SE	7.102	10.523	10.485
		p	0.92	0.6	0.23
	oxLDL	Estimate	1.120	0.541	0.848
		SE	1.053	1.977	1.324
		p	0.29	0.79	0.53
	TC/HDLc	Estimate	144.03	264.120	-100.79
		SE	231.98	347.991	345.11
		p	0.54	0.45	0.77
	log(Trig/HDLc)	Estimate	-80.94	-121.937	-17.79
		SE	76.12	120.629	102.14
		p	0.29	0.32	0.86
	LDLc/HDLc	Estimate	-161.61	-263.108	83.62
		SE	236.91	354.851	356.89
		p	0.497	0.46	0.82
	oxLDL/LDLc	Estimate	45.93	-0.622	49.86
		SE	114.11	213.822	142.81
		p	0.69	0.99	0.73
Total DMR	TC	Estimate	-0.195	0.089	0.123
		SE	0.628	0.957	0.815
		p	0.76	0.93	0.88
	log(Trig)	Estimate	5.679	0.456	5.058
		SE	8.779	13.825	10.778
		p	0.52	0.97	0.64
	HDLc	Estimate	0.205	0.165	-0.100
		SE	0.620	0.948	0.797
		p	0.74	0.86	0.9
	LDLc	Estimate	0.221	-0.187	-0.056
		SE	0.640	0.975	0.829
		p	0.73	0.85	0.95
	oxLDL	Estimate	-0.084	-0.005	-0.099
		SE	0.098	0.191	0.105
		p	0.4	0.98	0.35
	TC/HDLc	Estimate	-12.717	0.502	-9.995
		SE	20.535	31.678	26.978
		p	0.54	0.99	0.71
	log(Trig/HDLc)	Estimate	6.711	1.224	9.528
		SE	6.711	10.916	7.931
		p	0.32	0.91	0.24
	LDLc/HDLc	Estimate	12.103	-4.789	9.464
		SE	21.016	32.494	27.934
		p	0.57	0.88	0.74

		Total	Males	Females
oxLDL/LDLc	Estimate	-11.044	1.309	*-21.519*
	SE	10.346	19.597	*11.520*
	p	0.29	0.95	*0.071*

ABAS-II: Adaptive Behavior Assessment Scale Second Edition; DMR: Dementia Questionnaire for People with Learning Disabilities; HDL: high-density lipoprotein; K-BIT: Kaufman Brief Intelligence Test; LDL: low density lipoprotein; oxLDL: oxidized LDL; SSP: Spatial Span; TC, total cholesterol; ToLDx: Tower of London from Drexel University; Trig, triglycerides.

Supplementary Table 2: Changes from baseline in lipid concentrations, stratified by treatment and by sex.

		Total			Males			Females		
	Month	**Placebo**	**EGCG**	**p**	**Placebo**	**EGCG**	**p**	**Placebo**	**EGCG**	**p**
TC (mg/dL)	3	3.2±25.4	-5.5±23.4	0.3	8.1±24.6	-12.8±16.6	**0.014**	-1.2±25.9	3.0±27.5	0.4
	6	5.1±26.0	-6.5±24.2	0.13	13.9±28.9	-9.9±21.4	**0.011**	-3.4±20.2	-2.7±27.1	0.7
	12	1.9±17.2	-3.3±23.3	0.15	8.9±15.3	-8.1±18.9	**0.007**	-4.9±16.5	2.1±26.9	0.6
Triglycerides (mg/dL)	3	12.3±30.5	6.2±24.4	0.6	20.7±25.5	1.6±27.9	0.1	4.8±33.3	11.5±19.0	0.4
	6	11.1±25.3	-0.0±21.7	0.4	19.1±26.1	-0.1±24.9	*0.075*	3.5±22.6	0.0±18.0	0.6
	12	13.6±25.6	4.6±27.4	0.3	23.3±28.1	0.3±20.7	**0.047**	3.8±18.8	9.4±33.4	0.6
HDLc (mg/dL)	3	-1.7±6.4	-0.4±5.4	0.6	-0.5±7.1	-1.6±4.2	0.2	-2.7±5.7	0.9±6.3	*0.066*
	6	-1.7±7.2	-1.4±6.6	0.9	-0.3±7.3	-1.4±5.2	0.2	-3.2±7.0	-1.4±7.9	0.16
	12	-0.8±7.4	-4.7±8.4	0.2	-0.3±5.9	-4.8±6.1	**0.02**	-1.3±8.7	-4.6±10.7	0.8
LDLc (mg/dL)	3	4.9±27.7	-8.9±16.9	**0.03**	9.7±35.1	-11.5±15.9	**0.04**	0.5±18.5	-5.8±18.1	0.3
	6	7.1±28.6	-7.7±14.7	**0.004**	15.6±36.9	-8.5±17.7	**0.02**	-0.9±14.7	-6.8±10.9	0.13
	12	2.7±24.9	-1.5±14.7	**0.02**	9.8±29.3	-3.3±16.3	**0.03**	-4.4±17.8	0.4±12.9	0.5
oxLDL (U/L)	3	1.3±9.1	-4.0±8.1	**0.017**	-0.9±6.6	-6.3±6.1	**0.019**	3.2±10.7	-1.1±9.5	0.22
	6	-0.1±7.2	-3.9±7.5	**0.005**	-0.2±6.7	-6.4±6.9	**0.004**	0.0±7.8	-1.2±7.4	0.22
	12	-1.4±11.9	-2.3±11.6	**0.03**	-1.2±10.4	-3.4±10.2	**0.026**	-1.6±13.7	-1.1±13.2	0.4
TC/HDLc	3	0.2±0.6	-0.05±0.6	0.13	0.2±0.5	-0.1±0.3	0.12	0.2±0.7	0.02±0.8	0.4
	6	0.2±0.5	-0.01±0.5	**0.04**	0.3±0.9	-0.08±0.4	**0.02**	0.1±0.4	0.07±0.6	0.4

	12	0.2±0.8	0.4±0.7	0.5	0.3±0.6	0.4±0.6	0.3	0.09±0.9	0.5±0.8	0.99
LDLc/HDLc	3	0.1±0.5	-0.2±0.4	**0.014**	0.06±0.4	-0.1±0.3	0.23	0.2±0.6	-0.2±0.5	**0.049**
	6	0.2±0.4	-0.1±0.4	**0.002**	0.2±0.4	-0.1±0.3	**0.04**	0.1±0.4	-0.08±0.4	**0.03**
	12	0.1±0.7	0.3±0.8	0.2	0.2±0.5	0.4±0.6	0.5	0.06±0.8	0.3±0.6	0.4
Trig/HDLc	3	0.3±0.8	0.2±0.7	0.4	0.4±0.8	0.2±0.9	0.4	0.2±0.9	0.2±0.4	0.8
	6	0.3±0.6	0.09±0.6	0.25	0.4±0.6	0.1±0.7	0.3	0.2±0.5	0.05±0.5	0.6
	12	0.3±0.7	0.3±0.8	0.4	0.5±0.8	0.3±0.6	0.3	0.2±0.7	0.4±0.9	0.9
oxLDL/LDLc	3	0.002±0.05	-0.008±0.07	0.6	-0.02±0.06	-0.02±0.06	0.97	0.02±0.05	0.005±0.08	0.5
	6	-0.01±0.06	-0.004±0.07	0.9	-0.03±0.07	-0.03±0.06	0.98	0.006±0.05	0.02±0.07	0.9
	12	-0.01±0.1	-0.02±0.1	0.9	-0.02±0.1	-0.02±0.08	0.98	0.003±0.1	-0.02±0.1	0.9

HDL: high-density lipoprotein; LDL: low density lipoprotein; oxLDL: oxidized LDL; TC: Total Cholesterol; Trig, triglycerides.

Supplementary Table 3: Changes from baseline in executive function variables, stratified by treatment and sex.

		Total			Males			Females		
	Month	**Placebo**	**EGCG**	**p**	**Placebo**	**EGCG**	**p**	**Placebo**	**EGCG**	**p**
SSP Span Length	3	0.4±0.9	0.1±1.2	0.15	0.6±1.1	0.2±1.2	0.7	0.3±0.7	0.1±1.3	0.14
	6	0.2±1.4	0.4±1.2	0.8	0.2±1.6	0.7±1.3	0.4	0.1±1.3	0±1.1	0.2
	12	0.6±1.3	0.5±1.0	0.7	0.9±1.4	0.4±1.2	0.6	0.8±1.0	0.5±0.9	0.3
SSP Backward Span Length	3	0.2±1.2	0.1±1.2	0.8	0.0±1.2	-0.05±1.2	0.9	0.4±1.2	0.2±1.3	0.96
	6	0.2±1.3	0.4±1.5	0.4	-0.2±1.4	0.5±1.7	0.4	0.5±1.2	0.4±1.3	0.8
	12	0.3±1.4	0.3±1.5	0.5	0.1±1.0	0.5±1.5	0.3	0.6±1.7	0.2±1.6	0.9
ToLDx Solving Time	3	-156.6±240.7	-80±230.7	0.2	-119.5±246.6	-61.2±214.8	0.3	-185.9±238.4	-101±252.2	0.6
	6	-130.3±302.8	-163.5±266.4	0.8	-85.8±373.7	-163.6±231.8	0.8	-169.9±226.4	-163.5±306.1	0.98
	12	-235.8±250.1	-161.1±297.4	0.6	-251.9±294.1	-89.8±268.1	0.4	-222.4±214.6	-236.7±315.9	0.8
ToLDx Total Correct Movements	3	0.7±1.1	1.0±1.7	0.7	0.9±1.1	1.0±1.5	0.8	0.6±1.2	1.1±2.0	0.4
	6	1.0±1.3	1.3±1.5	0.6	1.0±1.4	1.2±1.3	0.94	0.9±1.3	1.4±1.7	0.27
	12	1.2±1.9	1.0±1.6	0.9	0.7±1.7	1.2±1.4	0.9	1.6±2.0	0.8±1.8	0.94
ToLDx Total Movements	3	-18.8±36.2	-17.5±34.6	0.5	-25.3±36.6	-16.1±33.9	0.2	-13.6±36.1	-19.2±36.4	0.5
	6	-9.6±42.4	-22.7±36.2	0.8	-10.9±52.6	-23.8±40.9	0.5	-8.4±32.3	-21.6±31.7	0.2
	12	-15.4±36.1	-20.9±32.0	0.6	-11.7±41.9	-14.2±35.5	0.7	-18.5±31.4	-28.2±26.9	0.16
ABAS-II	3	-1.9±7.4	5.9±11.5	**0.003**	-2.5±7.8	7.6±13.8	**0.03**	-1.4±7.1	3.8±7.5	**0.04**

Functional Academics	6	-1.7±9.0	6.1±10.3	**0.0005**	-1.8±10.0	7.8±12.7	**0.03**	-1.6±8.3	4.2±6.5	**0.006**
	12	-0.1±9.2	4.6±10.8	**0.002**	-1.1±10.9	6.4±11.3	**0.03**	0.9±7.0	2.7±10.2	**0.03**
ABAS-II Self-care	3	1.6±5.9	0.7±4.9	0.36	1.4±5.7	0.7±5.1	0.4	1.8±6.3	0.7±4.9	0.6
	6	2.3±5.7	2.0±5.0	0.4	2.5±5.2	1.5±5.9	0.3	2.1±6.2	2.7±3.8	0.9
	12	2.1±5.6	1.0±11.0	0.36	2.2±5.4	-0.8±14.9	0.2	2±6.1	2.8±3.3	0.9

ABAS-II: Adaptive Behavior Assessment Scale Second Edition; DMR: Dementia Questionnaire for People with Learning Disabilities; SSP: Spatial Span; ToLDx: Tower of London from Drexel University.

Supplementary Table 4: Longitudinal associations between the pooled changes from 0 to 3, 3 to 6, and 6 to 12 months, stratified by treatment and sex.

			Placebo			EGCG		
			Total	**Males**	**Females**	**Total**	**Males**	**Females**
SSP Span Length	TC	Estimate	0.053	0.103	-0.123	-0.016	0.121	-0.016
		SE	0.074	0.115	0.101	0.011	0.095	0.012
		p	0.48	0.38	0.23	0.15	0.21	0.18
	log(Triglycerides)	Estimate	-0.134	-0.487	1.358	0.030	*-2.814*	0.858
		SE	1.086	1.686	1.448	0.417	*1.517*	0.712
		p	0.9	0.77	0.36	0.94	*0.073*	0.24
	HDLc	Estimate	-0.068	-0.093	0.107	*0.032*	-0.131	0.046
		SE	0.077	0.128	0.102	*0.018*	0.095	0.029
		p	0.38	0.47	0.3	*0.084*	0.18	0.12
	LDLc	Estimate	-0.054	-0.118	0.136	**0.026**	-0.097	*0.029*
		SE	0.074	0.115	0.102	**0.012**	0.092	*0.014*
		p	0.47	0.31	0.19	**0.032**	0.3	*0.053*
	oxLDL	Estimate	0.000	-0.001	0.015	0.004	-0.010	0.007
		SE	0.012	0.022	0.015	0.012	0.020	0.016
		p	0.97	0.96	0.33	0.75	0.63	0.64
	TC/HDLc	Estimate	-0.218	2.196	-2.220	*-0.620*	2.432	-0.643
		SE	2.042	4.410	2.153	*0.369*	2.661	0.416
		p	0.92	0.62	0.31	*0.097*	0.37	0.13
	log(Trig/HDLc)	Estimate	0.672	0.276	0.552	-0.257	*-2.025*	0.566

			Placebo			EGCG		
			Total	**Males**	**Females**	**Total**	**Males**	**Females**
		SE	0.724	1.457	0.854	0.385	*1.135*	0.699
		p	0.36	0.85	0.52	0.51	*0.084*	0.42
	LDLc/HDLc	Estimate	0.196	-2.786	2.763	*0.772*	-1.783	0.572
		SE	2.100	4.387	2.251	*0.421*	2.591	0.520
		p	0.93	0.53	0.23	*0.07*	0.5	0.28
	oxLDL/LDLc	Estimate	0.475	-0.543	1.783	0.398	0.220	-0.001
		SE	1.384	2.116	1.746	1.301	2.220	1.710
		p	0.73	0.8	0.32	0.76	0.92	0.99
SSP Backward Span Length	TC	Estimate	-0.020	0.029	-0.120	0.003	-0.051	0.004
		SE	0.085	0.132	0.124	0.012	0.137	0.011
		p	0.82	0.83	0.34	0.83	0.71	0.7
	log(Triglycerides)	Estimate	0.250	-0.083	0.977	-0.095	1.066	0.041
		SE	1.256	1.939	1.791	0.493	2.310	0.694
		p	0.84	0.97	0.59	0.85	0.65	0.95
	HDLc	Estimate	0.007	-0.027	0.108	0.026	0.103	-0.002
		SE	0.089	0.147	0.126	0.022	0.138	0.028
		p	0.94	0.85	0.4	0.23	0.46	0.95
	LDLc	Estimate	0.017	-0.050	0.136	0.003	0.055	0.001
		SE	0.086	0.132	0.126	0.014	0.133	0.013
		p	0.84	0.71	0.29	0.82	0.68	0.94
	oxLDL	Estimate	-0.011	0.008	-0.017	0.000	-0.009	-0.001
		SE	0.014	0.025	0.019	0.014	0.030	0.015

			Placebo			EGCG		
			Total	**Males**	**Females**	**Total**	**Males**	**Females**
		p	0.43	0.76	0.37	0.98	0.77	0.94
	TC/HDLc	Estimate	*-4.234*	-3.312	*-5.066*	0.161	-3.593	0.262
		SE	*2.346*	5.083	*2.626*	0.423	3.801	0.367
		p	*0.076*	0.52	*0.063*	0.71	0.35	0.48
	log(Trig/HDLc)	Estimate	1.364	1.455	0.951	-0.452	1.065	-0.032
		SE	0.831	1.679	1.037	0.453	1.741	0.656
		p	0.11	0.39	0.37	0.32	0.54	0.96
	LDLc/HDLc	Estimate	*4.308*	3.044	*5.507*	-0.177	3.242	-0.110
		SE	*2.413*	5.058	*2.745*	0.479	3.624	0.465
		p	*0.079*	0.55	*0.053*	0.71	0.38	0.81
	oxLDL/LDLc	Estimate	0.270	0.911	-0.384	0.142	0.088	-0.068
		SE	1.599	2.440	2.164	1.536	3.384	1.556
		p	0.87	0.71	0.86	0.93	0.98	0.97
ToLDx Solving Time	TC	Estimate	*-29.148*	-35.660	-27.609	1.265	-14.350	2.083
		SE	*17.063*	22.495	30.957	1.879	20.792	1.812
		p	*0.093*	0.13	0.38	0.5	0.5	0.26
	log(Triglycerides)	Estimate	*454.089*	514.755	526.681	-124.370	5.169	13.238
		SE	*241.266*	332.256	407.753	75.002	322.997	113.072
		p	*0.065*	0.13	0.21	0.1	0.99	0.91
	HDLc	Estimate	28.898	42.309	23.963	2.474	15.588	3.126
		SE	17.900	25.704	31.585	3.270	20.695	4.376
		p	0.11	0.11	0.45	0.45	0.46	0.48

			Placebo			EGCG		
			Total	**Males**	**Females**	**Total**	**Males**	**Females**
	LDLc	Estimate	*29.407*	34.434	28.658	0.324	14.656	1.654
		SE	*17.114*	22.405	30.929	2.170	19.656	2.382
		p	*0.091*	0.14	0.360	0.88	0.46	0.49
	oxLDL	Estimate	-0.137	-0.344	-0.690	0.651	-1.826	1.871
		SE	2.683	4.062	3.892	2.118	4.158	2.457
		p	0.96	0.93	0.86	0.76	0.66	0.45
	TC/HDLc	Estimate	-757.793	-571.875	-821.472	35.478	-306.948	56.553
		SE	504.740	878.116	644.909	64.324	507.128	65.705
		p	0.14	0.52	0.21	0.58	0.55	0.4
	log(Trig/HDLc)	Estimate	*290.045*	166.939	*403.911*	**-148.838**	-64.522	4.151
		SE	*166.344*	285.470	*219.155*	**68.941**	225.960	117.260
		p	*0.086*	0.56	*0.07*	**0.035**	0.78	0.97
	LDLc/HDLc	Estimate	771.325	582.432	830.175	-10.999	330.032	-51.263
		SE	513.177	882.560	661.120	75.252	480.210	88.268
		p	0.14	0.52	0.22	0.88	0.5	0.57
	oxLDL/LDLc	Estimate	256.127	-27.329	466.591	-23.741	-173.763	-72.806
		SE	290.601	409.201	433.850	231.238	452.626	273.248
		p	0.38	0.95	0.29	0.92	0.7	0.79
ToLDx Total Correct Movements	TC	Estimate	0.159	-0.007	**0.039**	-0.019	-0.050	-0.021
		SE	0.108	0.155	**0.173**	0.015	0.125	0.017
		p	0.15	0.96	**0.032**	0.22	0.69	0.23
	log(Triglycerides)	Estimate	-2.020	0.207	**-4.976**	**1.222**	2.492	0.074

			Placebo			EGCG		
			Total	**Males**	**Females**	**Total**	**Males**	**Females**
		SE	1.525	2.290	**2.280**	**0.588**	2.057	1.061
		p	0.19	0.93	**0.037**	**0.042**	0.24	0.94
	HDLc	Estimate	-0.168	0.001	**-0.392**	0.036	0.068	0.042
		SE	0.113	0.177	**0.177**	0.026	0.126	0.041
		p	0.14	0.99	**0.034**	0.17	0.6	0.31
	LDLc	Estimate	-0.155	0.016	**-0.386**	-0.012	0.034	-0.029
		SE	0.108	0.154	**0.173**	0.017	0.121	0.022
		p	0.16	0.92	**0.033**	0.49	0.78	0.21
	oxLDL	Estimate	0.028	-0.009	**0.049**	-0.008	-0.015	-0.006
		SE	0.017	0.028	**0.022**	0.017	0.028	0.023
		p	0.11	0.74	**0.032**	0.63	0.6	0.79
	TC/HDLc	Estimate	*6.157*	0.347	**8.452**	-0.451	0.932	-0.517
		SE	*3.183*	5.903	**3.720**	0.513	3.419	0.594
		p	*0.058*	0.95	**0.03**	0.38	0.79	0.93
	log(Trig/HDLc)	Estimate	*-1.763*	0.068	*-2.405*	**1.393**	1.316	0.178
		SE	*1.049*	1.919	*1.264*	**0.549**	1.534	1.060
		p	*0.098*	0.97	*0.066*	**0.014**	0.4	0.87
	LDLc/HDLc	Estimate	-5.943	-0.394	**-8.136**	-0.628	-1.739	-0.831
		SE	3.236	5.933	**3.814**	0.600	3.259	0.798
		p	*0.071*	0.95	**0.041**	0.30	0.6	0.31
	oxLDL/LDLc	Estimate	2.567	-0.402	**5.163**	-1.105	-3.095	-0.110
		SE	1.832	2.751	**2.503**	1.831	3.074	2.471

			Placebo			EGCG		
			Total	**Males**	**Females**	**Total**	**Males**	**Females**
		p	0.17	0.88	**0.047**	0.55	0.32	0.96
ToLDx Total Movements	TC	Estimate	*-4.776*	-3.599	**-8.210**	-0.185	-0.457	-0.133
		SE	*2.488*	3.816	**3.882**	0.281	2.623	0.295
		p	*0.06*	0.36	**0.043**	0.51	0.86	0.66
	log(Triglycerides)	Estimate	*68.603*	48.541	**123.053**	-16.684	-22.158	1.459
		SE	*35.185*	56.361	**51.127**	11.123	43.026	18.408
		p	*0.056*	0.4	**0.022**	0.14	0.61	0.94
	HDLc	Estimate	*4.853*	4.583	*7.753*	0.763	0.740	0.794
		SE	*2.610*	4.360	*3.960*	0.490	2.643	0.712
		p	*0.068*	0.3	*0.059*	0.12	0.78	0.27
	LDLc	Estimate	*4.555*	3.264	**7.960**	0.504	0.968	0.512
		SE	*2.496*	3.801	**3.878**	0.324	2.531	0.388
		p	*0.073*	0.4	**0.049**	0.13	0.7	0.2
	oxLDL	Estimate	0.237	0.451	0.097	0.371	-0.391	0.625
		SE	0.391	0.689	0.488	0.315	0.585	0.400
		p	0.55	0.52	0.84	0.24	0.51	0.13
	TC/HDLc	Estimate	*-131.830*	5.066	**-193.256**	-9.061	-114.233	-5.884
		SE	*73.024*	147.277	**78.952**	9.766	70.139	10.498
		p	*0.076*	0.97	**0.02**	0.36	0.11	0.58
	log(Trig/HDLc)	Estimate	*44.629*	-8.105	**73.448**	**-22.797**	12.983	0.958
		SE	*24.066*	47.879	**26.830**	**10.452**	31.474	18.735
		p	*0.069*	0.87	**0.01**	**0.033**	0.68	0.96

			Placebo			EGCG		
			Total	**Males**	**Females**	**Total**	**Males**	**Females**
	LDLc/HDLc	Estimate	*129.926*	-3.934	**189.780**	17.053	*119.575*	10.556
		SE	*74.245*	148.022	**80.937**	11.412	*66.842*	14.103
		p	*0.085*	0.98	**0.025**	0.14	*0.083*	0.46
	oxLDL/LDLc	Estimate	*72.415*	39.162	*103.534*	20.801	-24.723	25.537
		SE	*42.043*	68.631	*53.113*	34.834	63.068	43.657
		p	*0.09*	0.57	*0.06*	0.55	0.7	0.56
ABAS-II Functional Academics	TC	Estimate	-0.976	-1.537	-0.491	0.095	*1.975*	0.095
		SE	0.597	0.947	0.859	0.097	*0.996*	0.084
		p	0.11	0.12	0.57	0.33	*0.056*	0.27
	log(Triglycerides)	Estimate	11.940	16.806	8.959	-4.476	**-38.202**	1.187
		SE	8.651	13.589	12.392	3.858	**16.309**	5.357
		p	0.17	0.23	0.48	0.25	**0.026**	0.83
	HDLc	Estimate	*1.065*	1.616	0.559	-0.003	*-1.935*	0.077
		SE	*0.619*	1.036	0.873	0.168	*1.001*	0.210
		p	*0.09*	0.13	0.53	0.99	*0.062*	0.72
	LDLc	Estimate	0.845	1.399	0.349	-0.122	**-2.020**	-0.031
		SE	0.600	0.939	0.870	0.108	**0.963**	0.103
		p	0.16	0.15	0.69	0.26	**0.044**	0.77
	oxLDL	Estimate	**0.300**	0.215	**0.277**	-0.026	0.103	-0.103
		SE	**0.100**	0.173	**0.133**	0.112	0.218	0.121
		p	**0.0038**	0.22	**0.046**	0.82	0.64	0.4
	TC/HDLc	Estimate	-8.270	28.024	-18.658	2.919	42.512	2.610

			Placebo			EGCG		
			Total	**Males**	**Females**	**Total**	**Males**	**Females**
		SE	16.256	35.063	17.889	3.262	27.475	2.844
		p	0.61	0.43	0.31	0.37	0.13	0.37
	log(Trig/HDLc)	Estimate	0.824	-13.600	9.724	-3.117	*-21.562*	0.254
		SE	5.781	11.608	7.119	3.453	*12.174*	5.047
		p	0.89	0.25	0.18	0.37	*0.09*	0.96
	LDLc/HDLc	Estimate	8.612	-24.528	16.715	-5.457	-43.121	-5.124
		SE	16.720	34.863	18.700	3.712	26.362	3.622
		p	0.61	0.49	0.38	0.15	0.11	0.17
	oxLDL/LDLc	Estimate	**32.176**	*30.587*	**34.432**	-4.615	15.122	-15.959
		SE	**11.318**	*17.244*	**14.915**	12.025	24.530	12.536
		p	**0.006**	*0.087*	**0.028**	0.7	0.54	0.21
ABAS-II Self-care	TC	Estimate	-0.414	-0.555	-0.496	0.027	-0.017	0.024
		SE	0.349	0.511	0.536	0.080	0.949	0.047
		p	0.24	0.29	0.36	0.73	0.99	0.61
	log(Triglycerides)	Estimate	6.589	6.948	7.826	-2.301	1.721	-2.557
		SE	5.043	7.274	7.718	3.109	15.537	2.806
		p	0.2	0.35	0.32	0.46	0.91	0.37
	HDLc	Estimate	0.494	0.567	0.619	0.170	0.486	-0.077
		SE	0.363	0.565	0.544	0.137	0.953	0.114
		p	0.18	0.33	0.26	0.22	0.61	0.51
	LDLc	Estimate	0.397	0.578	0.445	-0.027	-0.029	-0.028
		SE	0.351	0.508	0.543	0.089	0.917	0.057

			Placebo			EGCG		
			Total	**Males**	**Females**	**Total**	**Males**	**Females**
		p	0.26	0.27	0.42	0.76	0.98	0.62
	oxLDL	Estimate	0.022	-0.134	0.123	-0.006	-0.042	-0.009
		SE	0.058	0.093	0.082	0.091	0.208	0.065
		p	0.7	0.16	0.14	0.94	0.84	0.89
	TC/HDLc	Estimate	-1.064	-8.971	0.557	1.103	7.205	0.889
		SE	9.577	20.532	11.814	2.668	25.342	1.582
		p	0.91	0.67	0.96	0.68	0.78	0.58
	log(Trig/HDLc)	Estimate	0.849	2.767	0.976	-2.681	-3.036	-2.388
		SE	3.358	6.519	4.671	2.765	11.229	2.659
		p	0.8	0.67	0.84	0.34	0.79	0.38
	LDLc/HDLc	Estimate	0.691	8.674	-1.344	-4.186	-14.071	-0.319
		SE	9.852	20.373	12.349	3.021	24.316	1.991
		p	0.94	0.67	0.91	0.17	0.57	0.87
	oxLDL/LDLc	Estimate	-2.563	-10.279	5.010	-3.854	-11.750	1.530
		SE	6.570	9.274	9.800	9.637	22.626	6.726
		p	0.7	0.28	0.61	0.69	0.61	0.82

ABAS-II: Adaptive Behavior Assessment Scale Second Edition; DMR: Dementia Questionnaire for People with Learning Disabilities; HDL: high-density lipoprotein; LDL: low density lipoprotein; oxLDL: oxidized LDL; SSP: Spatial Span; TC, total cholesterol; ToLDx: Tower of London from Drexel University; Trig, triglycerides.

4. CHAPTER II: Homocysteine Concentrations and Executive Functions.

4.1. Introduction

As previously explained (see **General Introduction**), individuals with DS have reduced concentrations of Hcy when compared to control population (159). This alteration of the concentrations is in part due to the overexpression of DYRK1A that leads to an increase of NQO1 activity **(Figure 14)** (49, 50). This has led to Hcy to be considered an indirect biomarker of DYRK1A activity (10, 13). Despite of this association between DYRK1A activity and Hcy concentrations in DS, the only study that has tried to determine the association between Hcy and cognition in DS had found that individuals with higher Hcy have lower IQs (160).

Both in general population and in individuals with DS, there are several potential modifiers of Hcy concentrations. On one hand there are the polymorphisms of the genes implicated in the One Carbon Metabolism **(Figure 14)**. In general population homozygotes for the T allele of methylenetetrahydrofolate reductase (MTHFR) C677T (57, 59, 60) and Val allele carriers of the Catechol-O-methyltransferase (COMT) Val158Met (59) polymorphisms have been identified as having higher Hcy concentrations, although in some reports no effect for the MTHFR genotype has been observed (161, 162). In individuals with DS, the association of MTHFR C677T and Hcy concentrations has been described as being the opposite with T carriers having lower concentrations (56), although this is still controversial (58). Other associations include higher Hcy concentrations for carriers of the G

allele of the 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR) A2756G polymorphism (56, 163), and for the G homozygotes of the 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR) A66G who were receiving folate and B vitamin supplements (58). Incidentally, folate and B vitamin have also been associated to Hcy concentrations, in this case higher folate and B12 vitamin plasma concentrations were associated to lower Hcy concentrations in general population (161, 162), and folic acid supplements also decreased Hcy plasma concentrations in DS (58). These associations with folate concentrations can be derived from diet, and adherence to MeDi has shown to decrease Hcy concentrations (57, 164, 165).

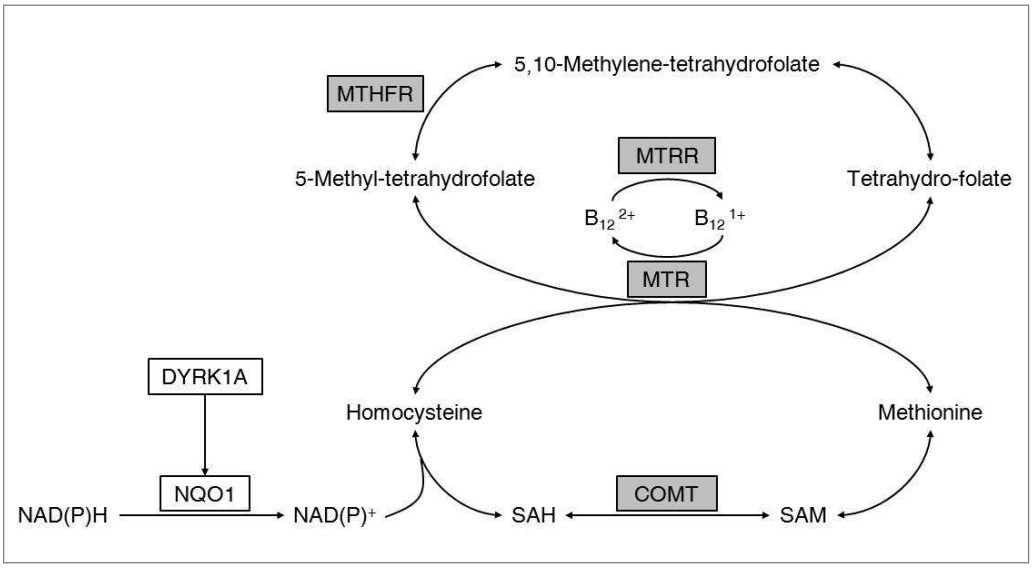

Figure 14: Folate and Hcy cycle. Boxes represent proteins/enzymes; grey boxes are for polymorphic genes. MTHFR: methylenetetrahydrofolate reductase; MTR: 5-methyltetrahydrofolate-homocysteine methyltransferase; MTRR: 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; COMT: catechol-O-methyltransferase; NQO1: NAD(P)H quinone dehydrogenase 1; DYRK1A: dual specificity tyrosine phosphorylation regulated kinase 1A.

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4.2. **Methods**

4.2.1. Participants

This study reports on baseline and longitudinal results of the TESDAD study population. Inclusion and exclusion criteria were similar to **Chapter I** (see **Annex 1**), with only those with the full trisomy being included in the analysis.

Controls were age-matched; those with Hcy concentrations above 25μmol/L were not included in the study.

This study followed the same ethical guidelines as **Chapter I**.

4.2.2. Neuropsychological test battery

The neuropsychological assessment was equivalent to the one described in **Chapter I**.

4.2.3. Adaptive behavior and dementia onset

The functional battery questionnaires were equivalent to the ones described in **Chapter I**.

4.2.4. Mediterranean Diet compliance questionnaire

The Mediterranean Diet questionnaires were completed as explained in **Chapter I**. No information on the diet could be recollected for 13 of the 74 individuals.

4.2.5. Homocysteine concentrations

Samples were handled as in **Chapter I**. Hcy concentration assessment was performed using the Immulite 1000 Systems (Siemens Healthcare, Spain) and the Immulite 1000 Homocysteine Kit as per provided protocol.

4.2.6. Genotyping

Genomic DNA was obtained as in **Chapter I**. COMTVal158Met allelic variants were determined as previously described (166). One of the individuals could not be genotyped.

Polymorphisms for MTHFR, MTRR, and MTR were genotyped via Taqman genotyping assays in a QuantStudio 12K Flex Real-Time PCR System (ThermoFisher) and analyzed with the supplied software. 2 individuals could not be genotyped for MTHFR, 3 for MTR, and 7 for MTRR.

4.2.7. Data analysis

Prior to statistical analyses, all variables were examined through various programs for accuracy of data entry and missing values. A descriptive analysis of the whole sample and separately for male and female study participants is provided by means of mean and standard deviation in the case of numeric variables and absolute and relative frequencies in the case of categorical variables, the IQ is presented as a median. The associations between Hcy concentrations and both the neurocognitive variables and functional executive variables of interest were studied by means of ANCOVA models at a multivariate level.

These models were adjusted for sex, MeDi compliance, and genetic polymorphisms, likelihood ratio tests were performed in order to discriminate between the adjusting variables that had an impact on the association between Hcy and cognition, in case the null hypothesis could be rejected, pairwise analysis were performed to analyze the differences between the groups.

ANCOVA models were fitted to analyze the effect of treatment in our variables of interest. Models were fitted for age, sex, and ApoE, and were further stratified by sex to analyze the specific effects of the treatment. Linear models stratified by treatment were fitted for changes in Hcy, age, and sex to study the association between changes in Hcy and changes in cognition.

Statistical significance was set at 0.05. No correction for multiple testing was performed as it is considered an exploratory study. All statistical analyses were performed using the statistical software package R (Version 3.1.3.; The R Foundation for Statistical Computing, Vienna, Austria).

4.3. **Results**

4.3.1. Descriptive demographics

74 individuals with DS were included in the study, of them 38 were male (51%), the mean age was 23.6 (SD±4.3), ranging from 16 to 34 years of age. Intellectual disability level was similar among male and female study participants, whereas mean Hcy levels were fairly larger among males, although the difference was not significant; see **Table 3**.

Table 3: Descriptive demographics of the study.

		Total (n=74)	Males (n=38, 51%)	Females (n=36, 49%)	p
Age	Mean ±SD	23.6 ±4.3	23.0 ±3.8	23.0 ±4.7	0.18
	Range	16-34	16-32	17-34	
IQ	Median	42.0	40.0	43.5	0.51
K-BIT	Mean ±SD	106.4 ±18.2	103.9 ±19.3	108.9 ±16.8	0.27
	Range	80-180	80-180	80-154	
Categorical IQ	≥40	43(58%)	20(53%)	23(64%)	0.3
	<40	31(42%)	18(47%)	13(36%)	
Hcy (µmol/L)	Mean ±SD	7.1 ±2.8	7.7 ±2.9	6.5 ±2.5	*0.054*
	Range	2.8-15.6	3.8-15.6	2.8-13.1	
MTRR genotype	A/A	17 (25%)	8 (24%)	9 (26%)	0.9
	A/G	37 (55%)	19 (58%)	18 (53%)	
	G/G	13 (19%)	6 (18%)	7 (21%)	
MTR genotype	A/A	50 (70%)	27 (77%)	23 (64%)	0.4
	A/G	19 (27%)	7 (20%)	12 (33%)	
	G/G	2 (3%)	1 (3%)	1 (3%)	
MTHFR genotype	G/G	28 (39%)	12 (33%)	16 (44%)	*0.08*
	G/A	31 (43%)	20 (56%)	11 (31%)	
	A/A	13 (18%)	4 (11%)	9 (25%)	
COMT genotype	A/A	6 (8%)	2 (5%)	4 (11%)	0.4
	A/G	25 (34%)	11 (30%)	14 (39%)	
	G/G	42 (58%)	24 (65%)	18 (50%)	
MeDi compliance	Yes (>7)*	45(74%)	24 (77%)	21 (70%)	0.5
	No (≤7)	16(26%)	7 (23%)	9 (30%)	

* refers to score in the MeDi questionnaire. Subjects with scores higher than 7 are considered compliant with the Mediterranean Diet. COMT: Catechol-O-methyltransferase; Hcy: homocysteine; IQ: Intellectual Quotient; K-BIT: Kaufman Brief Intelligence Test; MTHFR: Methylenetetrahydrofolate reductase; MTR: Methionine synthase; MTRR: 5-Methyltetrahydrofolate-Homocysteine Methyltransferase Reductase;

4.3.2. Differences between DS and controls

Age-matched controls were used to assess whether the concentrations of Hcy were decreased in our sample. 24 young adults, mean age 24.0±3.4 (19-33), 16 (67%) males and 8 (33%) females were used. The mean Hcy concentration was 11.1±2.4 μmol/L, with males having a mean of 11.9±2.4 μmol/L, and females 9.5±1.5 μmol/L. Females had significantly lower concentrations than males (Estimate=-2.181, 95% CI=[-4.238,-0.125], p=0.039).

ANCOVA models showed that controls had significantly higher concentrations than individuals with DS (Estimate=3.719, 95% CI=[2.491,4.948], p=3.45e-08) independently of sex. When stratifying for sex these differences stayed significant being higher in males (Estimate=4.117, 95% CI=[2.398,5.837], p=1.4e-05) than in females (Estimate=3.149, 95% CI=[1.269,5.029], p=0.0016) **(Figure 15)**.

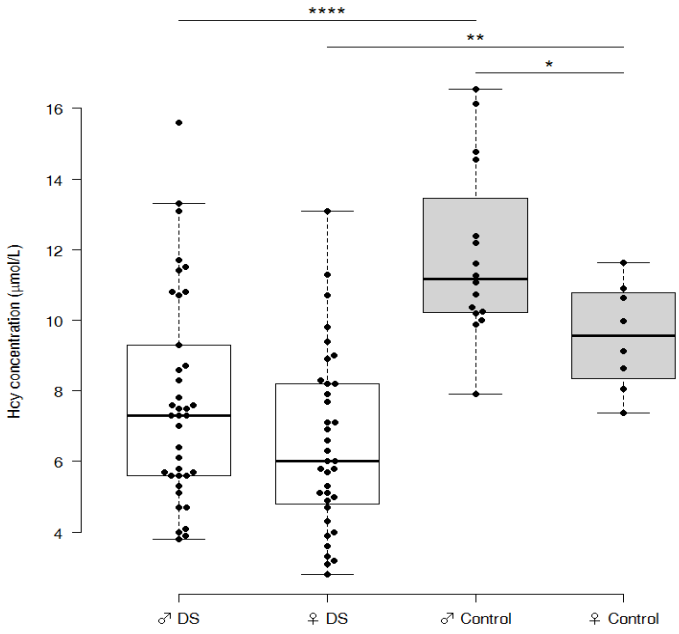

Figure 15: Hcy concentrations compared between individuals with DS (white) and controls (light grey).

4.3.3. Effect of genetic polymorphisms and MeDi compliance on Hcy concentrations

Models fitted for sex, age, and the genetic polymorphisms were performed to evaluate the effect of such variables on the concentrations of Hcy. Due to the distribution of MTR genotype the analysis was performed comparing A homozygotes with G carriers. No effect of the folate metabolism genotypes could be observed on Hcy concentrations as well as no differences in the pairwise analysis. There were no differences on MeDi compliance groups, either.

4.3.4. Associations between Hcy plasma concentrations and neurocognitive variables of EF

Models were fitted to evaluate the effects of Hcy concentrations on cognition, taking into account several other parameters including age, sex, and the genotypes of MTHFR, MTR, MTRR, and COMT. Likelihood ratio tests were performed to eliminate variables that were superfluous to the analysis, although sex and Hcy were always included, while age and MTHFR were not. Significant associations were found between Hcy and several ToLDx and ABAS-II variables; with individuals with higher concentrations of Hcy performing worse in ToLDx and having lower scores in ABAS-II. Similar non-significant associations were found for Digit Span, SVFT, and DMR **(Table 4)**.

Table 4: Results from the ANCOVA models.

	Estimate	SE	p
K-BIT	-0.382	0.703	0.59
Digits			
Forward Span	-0.038	0.033	0.26
Forward Score	-0.087	0.056	0.13
Reverse Span	-0.094	0.049	*0.058*
Reverse Score	-0.084	0.052	0.11
Total Score	-0.178	0.094	*0.062*
SVFT			
Total correct words	-0.312	0.186	*0.099*
ToLDx			
Number of solved items	-0.309	0.127	**0.018**
Total number of movements	2.966	1.553	*0.061*
Total solving time	33.25	11.95	**0.007**
Total number of problems with correct movements	-0.173	0.059	**0.004**
ABAS-II			
Communication	-0.825	0.459	*0.078*
Community Use	-1.156	0.560	**0.043**
Functional Academics	-1.233	0.733	*0.097*
Home Living	-1.006	0.497	**0.046**
Health and Safety	-0.799	0.382	**0.04**
Leisure	-0.634	0.459	0.17
Self-care	-0.978	0.314	**0.003**
Self-direction	-1.092	0.573	*0.06*
Social	-0.139	0.486	0.78
Total Score	-9.466	4.079	**0.023**
DMR			
Cognitive scores	0.319	0.188	*0.094*
Social Scores	0.181	0.167	0.28
Total Score	0.529	0.276	*0.06*

ABAS-II: Adaptive Behavior Assessment Scale Second Edition; DMR: Dementia Questionnaire for People with Learning Disabilities; K-BIT: Kaufman Brief Intelligence Test; p: p-value; SE: Standard Error; ToLDx: Tower of London from Drexel University

4.3.5. Effects of the One Carbon Metabolism polymorphisms and MeDi compliance on cognition

With the same models used to determine the association between Hcy and cognition, effects of specific genotypes could be observed on cognition. Such is the case of MTRR on ToLDx Total number of movements, although pairwise comparisons showed no significant differences between genotypes (A/G vs. A/A: Estimate=20.739, 95% CI=[-3.666,45.145], p=0.11; G/G vs. A/A: Estimate=-1.823, 95% CI=[-31.777,28.130], p=0.99; G/G vs. A/G: Estimate=-22.563, 95% CI=[-48.761,3.635], p=0.1). In the case of MTR significant differences could be observed between the G carriers and the A homozygotes in K-BIT (G/- vs. A/A: Estimate=8.523, 95% CI=[0.278,16.774], p=0.04), and ABAS-II Health and Safety (G/- vs. A/A: Estimate=-4.655, 95% CI=[-9.195,-0.115], p=0.045) **(Figure 16)**. According to these results, G carriers have higher intelligence, but lower functionality related to Health and Safety.

Neither sex nor age had any significant effect on any of the variables.

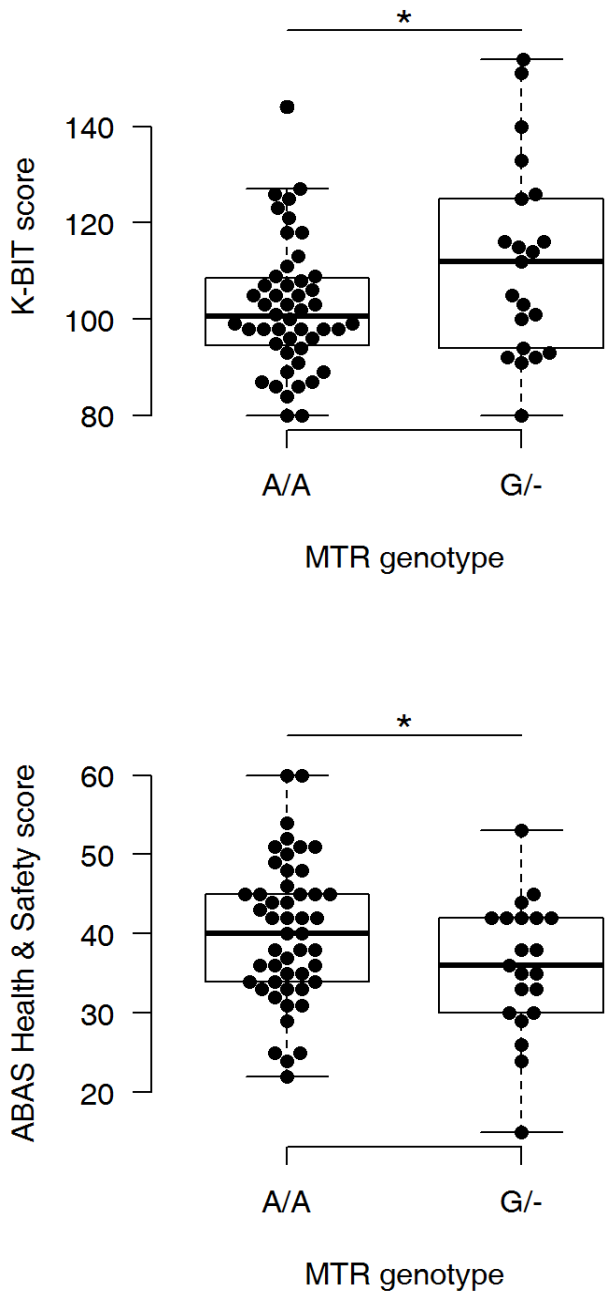

Figure 16: Differences between MTR genotypes and cognition. The left panel represents shows the differences between A homozygotes and G carriers in K-BIT scores. The right panel shows differences in ABAS-II Health and Safety scores between A homozygotes and G carriers.

MeDi had a significant effect on functional variables, those in the MeDi non-compliance group had lower scores in the ABAS-II Communication subscale (Estimate=-7.143, 95% CI=[-13.132, -1.1541], p=0.02), and higher scores in DMR Social Scores (Estimate=4.846, 95% CI=[2.637,7.056], p=0.0001) and the total DMR Scores (Estimate=6.054, 95% CI=[2.404,9.704], p=0.0016) **(Figure 17)**. Therefore, individuals who followed the Mediterranean diet had higher functionality and fewer signs of dementia.

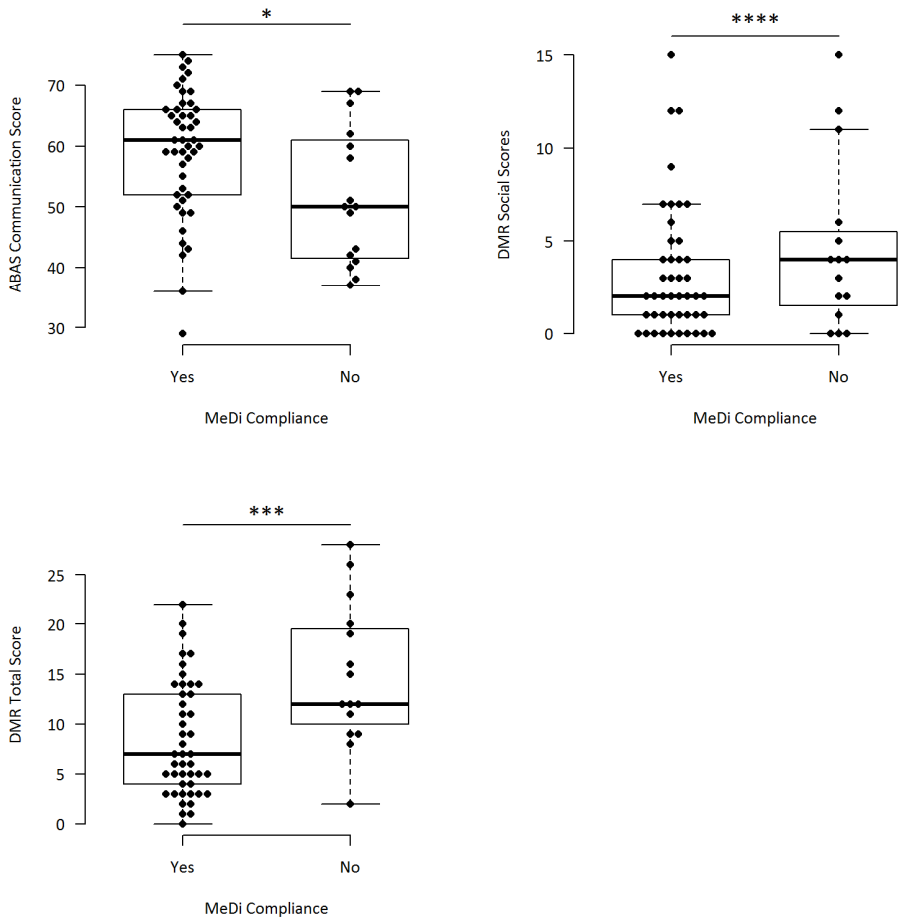

Figure 17: Differences in functionality depending on MeDi compliance. The panel on the left represents the differences in ABAS-II Communication Scores, the middle panel represents the differences DMR Social Scores, and the right panel represents the differences in DMR Total Scores.

4.3.6. Effects of EGCG on Hcy concentrations

As previously seen in the results of our clinical trial (Annex 1), there is an effect of treatment on the plasma concentrations of Hcy, with those in the EGCG group having a higher increase at 12 months than the ones in the placebo group (Estimate=0.589, SE=0.217, p=0.008) **(Figure 18)**. Age did not have an effect on the changes and the effect of sex was non-significant (Females vs. Males: Estimate=0.421, SE=0.225, p=0.07).

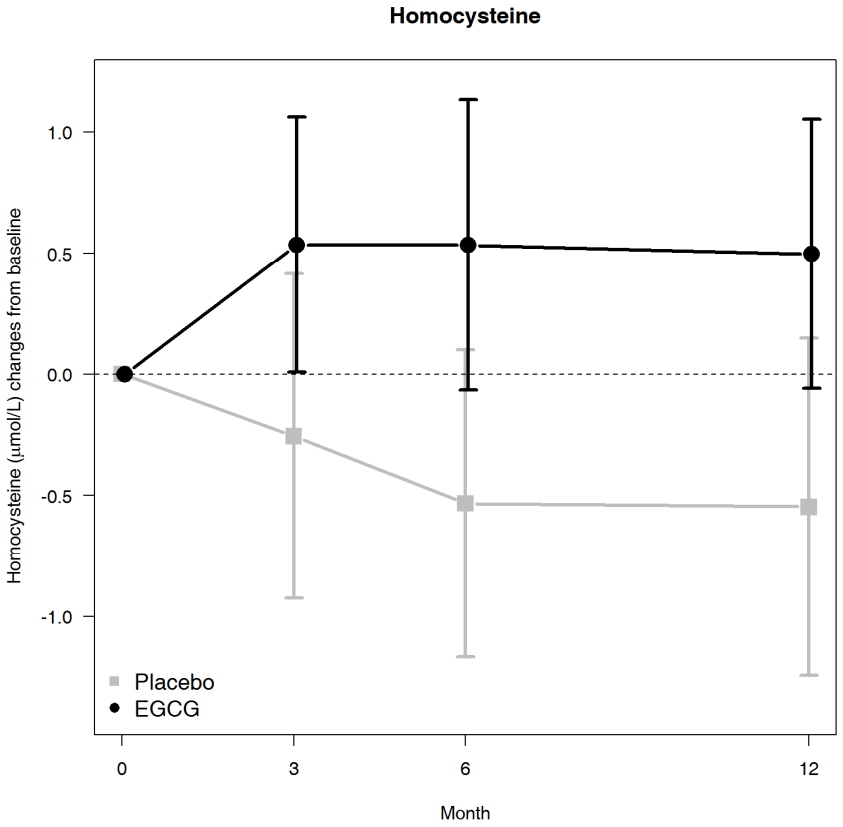

Figure 18: Changes in Homocysteine concentrations from baseline. On the top graph changes for the Placebo group are in light grey, while EGCG is in black.

4.3.7. Effects of EGCG on cognition

Similarly to what we have observed in **Chapter I**, the only studied variable in which we could observe an effect of treatment is the ABAS-II Functional Academics subscale (EGCG vs. Placebo: Estimate=3.313, SE=1.493, p=0.03) (for graph **see Chapter I Figure 13**).

4.3.8. Longitudinal Associations between changes in Hcy concentrations and changes in cognition

Models fitted for sex, age, and changes in Hcy concentrations stratified by treatment were performed in order to analyze the specific associations between changes in cognition and changes in Hcy concentrations. Results are summarized in **Table 5**. These results show how increases in Hcy concentrations in the Placebo group are associated to increases in both ToLDx solving time and total movements, reflecting, thus, worse planning skills. On the contrary, for the EGCG group only one association could be found, in this case increases in Hcy concentrations were associated to increases in the ABAS-II Community Use subscale.

Table 5: Results from the association models between changes in cognition and changes in Hcy, stratified by treatment.

		EGCG	Placebo
ToLDx solving time	Estimate	-8.264	**20.561**
	SE	9.905	**10.358**
	p	0.41	**0.05**
ToLDx total correct movements	Estimate	0.033	0.045
	SE	0.077	0.068
	p	0.67	0.51
ToLDx total movements	Estimate	-0.676	**2.966**
	SE	1.532	**1.481**
	p	0.66	**0.048**
ABAS-II Communication	Estimate	-0.097	-0.017
	SE	0.367	0.396
	p	0.79	0.97
ABAS-II Community Use	Estimate	**0.768**	-0.318
	SE	**0.387**	0.325
	p	**0.05**	0.33
ABAS-II Functional Academics	Estimate	0.086	-0.047
	SE	0.490	0.410
	p	0.86	0.91
ABAS-II Home Living	Estimate	-0.089	-0.243
	SE	0.406	0.270
	p	0.83	0.4
ABAS-II Health and Safety	Estimate	0.031	-0.172
	SE	0.379	0.308
	p	0.9	0.58
ABAS-II Self-care	Estimate	0.359	-0.352
	SE	0.417	0.228
	p	0.39	0.13
ABAS-II Self-direction	Estimate	0.219	0.119
	SE	0.409	0.358
	p	0.59	0.74
Total ABAS-II	Estimate	1.653	-1.263
	SE	2.437	1.713
	p	0.5	0.46
DMR Cognitive Scores	Estimate	-0.086	0.162
	SE	0.105	0.108
	p	0.4	0.14
Total DMR	Estimate	0.102	0.173
	SE	0.184	0.185
	p	0.56	0.35

ABAS-II: Adaptive Behavior Assessment Scale-II, DMR: Dementia Questionnaire for people with Learning Disabilities, ToLDx: Tower of London from Drexel University.

4.4. Discussion

Our study has found an inverse association between EF and Hcy concentrations in young DS adults, specifically in planning skills and daily live functionality. This association is seldom affected by the genetic polymorphisms described to modulate Hcy concentrations or by MeDi compliance, with only the MTR genotype having an effect on K-BIT scores and functionality and with MeDi having a possible protective effect on dementia signs.

4.4.1. Modifiers of Hcy concentrations

As previously described, individuals with DS had significantly lower concentrations of Hcy than controls (159). Similarly, males had higher concentrations than females (59), although this difference was only a trend in individuals with DS.

Although One Carbon Metabolism polymorphisms and diet have generally been described to have an influence on Hcy concentrations, some studies challenge such influence (58, 163, 167). Our results go in the same line of these latter studies, with neither polymorphisms nor MeDi compliance affecting Hcy concentrations.

4.4.2. Hcy associations with cognitive tests and functional variables

Hcy concentrations have shown to be associated with impaired EF in general population, including working memory, attention, planning, and mental flexibility (27, 29, 32, 35). In our population, higher concentrations of Hcy were also associated to worse performance in

planning skills which translate into worse scores in the ABAS-II questionnaires. Of the cognitive tasks analyzed, the one that had a stronger association with Hcy concentrations was ToLDx, which assesses complex planning-execution capability, showing that individuals with higher Hcy perform significantly worse in three of the aspects evaluated in this test, and, thus, confirm a poorer performance on tasks involving planning skills. Furthermore, several subscales of the ABAS-II which are tightly related with EF, such as Community use, Home Living, Health and Safety, Self-care, as well as the total score, had an important association with Hcy in the same direction of what observed in TOLDX, showing that Hcy concentrations are not only associated to lower performance in cognitive tasks, but that there is also an impact on everyday living. Furthermore, even though the association is non-significant, the fact that higher Hcy concentrations are associated to higher DMR scores is in accordance with Hcy being a risk factor for AD in the general population. Despite a previous study that found a correlation between higher Hcy and lower IQ in a DS population (160) we could not replicate such results.

Because several studies have proposed Hcy as a surrogate biomarker of DYRK1A activity, and DYRK1A excess of activity is known to be deleterious on cognitive performance in DS individuals, it could be expected that subjects with higher Hcy plasma concentrations performed better in the cognitive tests (10, 13). The fact that Ds individuals show hypohomocysteinemia and are intellectually impaired would support this assumption. However, as we have shown, subjects with higher Hcy plasma concentrations performed consistently worse.

This may suggest that downstream factors have a more direct effect on symptom progression.

There are several theories on how Hcy could be affecting cognitive performance, on one hand there is the possibility that Hcy concentrations are toxic on themselves (168, 169) by increasing oxidative stress in the brain, either by producing reactive oxygen species or by decreasing the endogenous antioxidant response (170). This increased oxidative state could lead to an increase in the permeability of the blood-brain barrier (171). Furthermore, hyperhomocysteinemia has a role in Alzheimer neuropathology, as it promotes Aβ and tau deposition (170), as well as increasing the atrophy generated by amyloid accumulation (169) Additionally, Hcy could act through other mechanisms, including the excessive promotion of calcium influx, the promotion of apoptosis, and the disruption of normal neurotransmission (31, 170), or the alteration of the vascular system (31, 169). In the case of DS, a model of methylation status suggested that higher oxidative stress renders higher Hcy concentrations, in which could be a feedback mechanism (159).

4.4.3. One Carbon Metabolism associations with cognition

A previous study seeking to understand the effect of both Hcy and its related polymorphisms on IQ, in individuals with DS, could only observe differences in IQ depending on the MTHFR C677T genotype, with T carriers having lower IQ (160). However, we did not observe any effect of the MTHFR on the associations of Hcy with

cognition. Of the other polymorphisms related to the One-Carbon Metabolism, only COMT did not have any effect on cognition, while G carriers of the MTR allele showed higher K-BIT scores than A homozygotes, as well as worse scores in the ABAS-II Health and Safety. MTRR polymorphism did have an effect on the association between Hcy and the total number of movements in ToLDx, but there were no significant differences between genotypes. However, all these associations need to be confirmed in larger populations.

4.4.4. MeDi compliance and associations with functionality

Similarly to what we observed in **Chapter I**, those individuals in the compliant group presented having lower scores of DMR Social and Total scores as well as higher scores in ABAS-II Communication subscale. These results are of interest as we have previously described that values of both DMR total score and the ABAS-II Communication subscale as being correlated with cognitive and peripheral markers of dementia (86).

4.4.5. Changes in Hcy, cognition, and their treatment-dependent associations

As previously shown in both the pilot study (10) and the Phase II clinical trial **(Annex 1)**, the treatment with EGCG increased Hcy concentrations in individuals with DS. This change in the concentrations is considered to be a biomarker of treatment efficacy (10, 13) due to the lowering effect on Hcy concentrations of the overexpression of DYRK1A (49, 50).

Additionally, EGCG has also proven its efficacy as an enhancer of cognition and functionality in population with DS (REF). In this case, as in **Chapter I**, a significant treatment effect could only be observed in the ABAS-II Functional Academics subscale.

Because of the associations observed in the baseline and the treatment effects we wanted to further explore whether Hcy could be a good biomarker of cognitive change. To this end we performed association models for the changes found during the 12 months of treatment. Our results show how, in the case of the placebo group, increases in Hcy concentrations are associated to worse performance in the ToLDx planning task, as it has already been observed in the basal conditions. For the EGCG group, the only association with changes in Hcy concentrations was for the ABAS-II Community Use subscale, with increases in Hcy concentrations being associated to higher scores in this subscale. However for this subscale there was no treatment effect. These results seem to point out that although there is a clear treatment effect on both Hcy concentrations and functionality that may be linked to the normalization of DYRK1A activity, there are other factors affecting both variables.

4.5. **Limitations**

Because neither oxidative stress nor folate intake were measured we are unable to evaluate the relationship between these two variables, Hcy plasma concentrations, and EF. The study of such relationship would be of importance to better understand the mechanism by which Hcy leads to worse EF in DS. In the same line, although we have not experimentally confirmed that DYRK1A activity is the same in all the

patients, because all of them carry the complete trisomy, we could assume that the differences in Hcy are almost negligible, and, therefore, that other factors are changing Hcy plasma concentrations, folate intake and oxidative stress may be important determinants. Additionally, the fact that we could not have all the dietary and genetic data for all the individuals has probably masked part of the effects of these variables.

In view of our results, studies in larger populations with DS could be of interest to understand how Hcy, and potentially its relationship with the oxidative status, affects EF and adaptive behavior throughout lifetime. Similarly to with **Chapter I**, due to our results regarding MeDi and DMR scores, a longitudinal study on the effects of MeDi compliance in this population is needed. Finally, longitudinal studies on how these associations are affected by the administration of compounds that selectively target DYRK1A are needed.

4.6. Conclusions

We found that higher Hcy concentrations are associated to worse EF, specifically to worse performance in planning skills and in daily living functionality in young adults with DS. These associations are important due to the deficits in EF and adaptive behavior in this population. However, we cannot conclude which is the source of such association, as several of the modifiers of Hcy concentrations were not measured.

Of the modifiers we could measure, none had an effect on Hcy plasma concentrations, although several did affect cognition (i.e. MTR genotype with cognition and functionality, and MeDi compliance with

a better functionality and lower signs of cognitive decline). This latter association suggests that MeDi compliance has a protective effect on the development of dementia in this population, as described in **Chapter I.**

We also observe that although the treatment had an effect on both Hcy concentrations and functionality, these changes were not associated to cognitive improvements in the treatment group. This would indicate that, despite Hcy being a promising cognitive function biomarker, it cannot be used as a biomarker of cognitive change due to treatment.

5. CHAPTER III: Years of Education and dementia markers

5.1. Introduction

Peripheral amyloid concentrations have been studied for their potential use as tools to predict dementia progression both in general population **(see General Introduction)** and in DS. In the case of DS, there is an increased peripheral concentrations of Aβ peptides compared to age matched controls (77-82, 85). These increased concentrations are related to the triplication of the APP gene which leads to an elevated production and accumulation of amyloid peptides (172), which leads not only to increased plasma amyloid concentrations, but to a buildup of amyloid plaques already by 40 years of age or less (173). Cognitive decline starts as early as 30 years of age, in some cases, with dementia onset being around 50 years, although not all individuals with DS develop AD (24).

The study of plasma amyloid concentrations and their association to dementia risk in old individuals with DS has shown the usefulness of such biomarkers. In particular, increases of $A\beta_{40}$ and decreases in $A\beta_{42}$ and the $A\beta_{42}/A\beta_{40}$ ratio are associated with conversion to dementia (85). Additionally, longitudinal studies show that subjects with higher $A\beta_{42}$ or $A\beta_{40}$ concentrations at baseline are at higher risk to develop dementia (83, 84). In a previous study we described an association between $A\beta_{42}$ concentrations and cognitive features characteristic of neurodegeneration in young adults with DS, such as a poorer performance in SVFT (lower number of correct words and lower number of switches) and higher scores in the DMR. These results

indicate that $A\beta_{42}$ can be considered as risk marker for early AD development in DS population (86).

AD risk clearly increases with age, but can be modified by factors such as ApoE genotype **(see General Introduction)** or education. The latter has been identified as a protective factor for both the incidence and the prevalence of dementia and AD in the general population (174-176). Besides, its effects are not limited to cognition as it is correlated with reduced Aβ plaque burden, measured through PIB retention (177), especially in populations at higher risk of AD such as ApoE ε4 carriers (178). Education modulates the concentrations of CSF t-tau (total-tau) and p-tau (phospho-tau), and the ratios of t-tau/$A\beta_{42}$ and p-tau/$A\beta_{42}$ (179). Contrary to its central effects, the potential influence of education on peripheral β-amyloid biomarkers is not clear. There is evidence showing a trend for subjects with lower literacy or shorter educational periods to have lower $A\beta_{40}$, $A\beta_{42}$ plasma concentrations or lower $A\beta_{42}/A\beta_{40}$ ratio (76, 180), while other studies have observed lower $A\beta_{42}/A\beta_{40}$ ratios in subjects with higher educational attainment (181, 182). These ratios have also been proposed to be predictors for cognitive decline (180, 182).

To our knowledge, only one study has sought to understand the effects of education in adults with DS; Temple *et al* observed that adults with DS (Mean age: 46, range: 29-67) who had attained higher level of schooling had less symptoms of dementia (183), even though the implications of education on peripheral β-amyloid biomarkers were not explored. In fact, the effect of years of education on cognition or amyloidosis biomarkers has not been studied in young population, possibly because cognitive decline is an age-related

process. Nevertheless, it is known that changes in biomarkers precede the changes in cognition (184) and education could have an effect on peripheral amyloid concentrations before affecting cognition, as the DS population has increased APP expression along life.

5.2. Methods

5.2.1. Participants

The sample was drawn from the baseline visit of the TESDAD study sample as in the previous chapters. From a cohort of 84 subjects 60 were enrolled in this cross-sectional study because they had reliable $A\beta_{40}$ plasma concentrations. Exclusion criteria were the same as described previously (**Chapter I** and **Chapter II**).

Ethical guidelines were as previously described (**Chapter I**).

5.2.2. Neuropsychological test battery

We used the SVFT (143) as a measure of semantic memory and executive functioning. These cognitive outcomes were fitted in our analysis according to their correlation with AD biomarkers we had observed in a previous study (86). Semantic memory was measured as the total number of correctly generated words in 60 seconds, and executive functioning by evaluating clustering and switching measures to determine the strategies used to perform the task. Definitions of clustering and switching have been previously described (185).

The intellectual quotient (IQ) was also estimated as previously (**Chapter I**). Subjects were further stratified in statistical analyses as displaying IQ $\geq$ or $<$ than 40.

5.2.3. Dementia onset

The DMR questionnaire was administered as detailed before (**Chapter I**).

5.2.4. Years of Education

The variable "years of education" was collected through a caregiver interview, taking into account years of regular school attendance in specialized or non-specialized educational centers.

5.2.5. Plasma amyloid peptides concentrations

Plasma samples were obtained as for both **Chapter I** and **Chapter II**, and handled equivalently. The analysis was performed as per manufacturer protocol (86) using the Inno-bia Plasma Aβ forms assay (Innogenetics, Fujirebio) read on a Bio-Plex 200 Systems (Bio-Rad) instrument. Quantification was performed with the provided software (Bioplex Manager 6.1).

Detection limits were between 5 and 420 pg/mL.

5.2.6. ApoE genotyping

ApoE genotyping was performed as described in **Chapter I**. Five of the 60 subjects could not be genotyped.

5.2.7. Data analysis

Results are described by means of measures of both central tendency (mean) and variability (standard deviation and range) for numeric variables, and absolute and relative frequencies for categorical

variables. In the case of the IQ, only the median is reported because no distinction is made of values below 40. The computation of all correlations of interest was done using either Pearson or Spearman correlation coefficients. ANCOVA models were used to study the associations in between YoE, other cognitive and functional measures, and AD biomarkers, on one hand, and sex, IQ, ApoE genotype, and age, on the other hand. For these analyses, the IQ was categorized into two groups: mild/moderate (IQ ≥ 40) and severe (IQ < 40) within the range of intellectual disability (ID) level.

Statistical significance was set at 0.05. All statistical analyses were performed using the statistical software package R (Version 3.1.3.; The R Foundation for Statistical Computing, Vienna, Austria).

5.3. **Results**

5.3.1. Descriptive demographics

Subjects were young adults (Mean age: 23.35 ±4.5 years old), with an equal distribution in sex (50% females), and most of them (95%) presented the full trisomy. The rest of demographic characteristics are summarized in **Table 6**. When comparing them according to sex, females were marginally older (p=0.049) than male individuals, but no other significant difference could be observed. The concentrations of $A\beta_{1\text{-}40}$ and $A\beta_{N\text{-}40}$ were directly correlated (R=0.718, p=0.00000009, 95% CI=[0.545,0.832]).

Table 6: Demographic description of the sample.

		Total (n=60)	Males (n=30)	Females (n=30)	p
Age (years)	Mean (±SD)	23.35±4.5	22.2±3.9	24.5±4.8	0.049
	Range	16-34	16-31	17-34	
IQ	Median	41.0	40.00	42.5	0.82
K-BIT score	Mean (±SD)	103.9±15.0	101.4±15.9	106.3±13.9	0.36
	Range	80.0-151.0	80.0-144.0	80.0-151.0	
Categorical IQ	<40	24 (40%)	14 (46.6%)	10 (33.3%)	0.29
	≥40	36 (60%)	16 (53.3%)	20 (66.6%)	
ApoE genotype	ε2/ε3	5 (9.1%)	2 (7.1%)	3 (11.1%)	0.74
	ε3/ε3	45 (81.8%)	24 (85.7%)	21 (77.7%)	
	ε3/ε4	5 (9.1%)	2 (7.1%)	3 (11.1%)	
Karyotype	Full Trisomy	57 (95%)	29 (96.6%)	28 (93.3%)	0.6
	Translocation	2 (3.3%)	1 (3.3%)	1 (3.3%)	
	Mosaicism	1(1.6%)	0 (0%)	1 (3.3%)	
YoE	Mean (±SD)	13.2±1.9	13.4±2.1	12.9±1.7	0.2
	Range	10-18	10-18	10-15	
DMR Sum of Cognitive scores (SCS)	Mean (±SD)	4.4±5.4	5.1±6.3	3.8±4.3	0.51
	Range	0-27	0-27	0-15	
DMR Sum of Social Scores (SOS)	Mean (±SD)	6.4±3.9	6.6±4.0	6.3±3.8	0.99
	Range	0-16	0-16	1-15	
DMR Total Score	Mean (±SD)	10.9±7.9	11.6±8.7	10.2±7.1	0.65
	Range	0-43	0-43	2-26	
SVFT Number of switches	Mean (±SD)	4.2±2.4	3.9±2.7	4.5±2.1	0.53
	Range	0-13	0-13	0-9	
SVFT Mean cluster size	Mean (±SD)	1.2±0.8	1.2±0.7	1.1±0.8	0.46
	Range	0-3.33	0-2.66	0-3.33	
SVFT Total correct words	Mean (±SD)	9.0±4.3	8.5±4.9	9.6±3.6	0.6
	Range	0-20	0-17	3-20	
$A\beta_{1-40}$ (pg/mL)	Mean (±SD)	265.6±49.5	258.4±43.5	273.0±54.7	0.31
	Range	174.0-439.3	174.0-344.7	184.2-439.3	
$A\beta_{N-40}$ (pg/mL)	Mean (±SD)	276.8±60.6	274.2±47.3	279.2±71.9	0.69
	Range	173.4-435.1	191.5-343.9	173.4-435.1	

Aβ: β-amyloid peptide; ApoE: Apolipoprotein E; DMR: Dementia Questionnaire for Persons with Intellectual Disability; IQ: Intellectual Quocient; K-BIT: Kaufman Brief Intelligence Test; SD: Standard Deviation; SVFT: Semantic Verbal Fluency Task; YoE: years of education.

5.3.2. Correlations between $A\beta_{40}$ plasma concentrations and DMR

As previously described, there was an effect of Categorical IQ on DMR Cognitive Scores, with those in the <40 IQ group having a higher mean score (≥40 vs. <40: Estimate=-4.802, SE=1.356, p=0.00084). Therefore, stratified analyses were performed. In the <40 group a significant direct correlation was found for $A\beta_{1-40}$ and Cognitive scores (rho=0.526, p=0.014, 95% CI=[0.130,0.800]), but not for $A\beta_{N-40}$ (rho=0.439, p=0.068, 95% CI=[-0.090,0.854]) (Fig. 1). In the ≥40 group no correlations were observed. Thus, lower DMR Cognitive scores in the <40 group correlated with decreased concentrations of $A\beta_{1-40}$.

5.3.3. Correlations of $A\beta_{40}$ plasma concentrations with SVFT measures

Similarly to what happened with YoE, only significant correlations with SVFT measures were observed in the IQ<40 group. In this case higher $A\beta_{40}$ concentrations correlate with worse performance in the SVFT **(Table 7)**.

Table 7: Correlations in the IQ<40 group between Aβ40 concentrations and the different variables of the SVFT.

		$A\beta_{1-40}$	**$A\beta_{N-40}$**
Number of switches	R	-0.484	-0.462
	p	**0.03**	*0.061*
	95% CI	[-0.763,-0.052]	[-0.771,0.023]
Total number of correct words	R	-0.499	-0.624
	p	**0.018**	**0.004**
	95% CI	[-0.760,-0.098]	[-0.840,-0.237]

CI: Confidence interval; p: p-value; R: Pearson's correlation.

5.3.4. Associations between YoE and DMR scores

Models adjusted for YoE, sex, age, and categorical IQ show an effect of YoE on DMR Cognitive scores (Estimate=-0.779, p=0.032, 95% CI=[-1.490,-0.067]), but no effect of YoE was observed on DMR Social or Total scores **(Figure 19)**.

The possible effects of sex, age, and Categorical IQ on DMR scores were also evaluated, but neither sex nor age had an effect on DMR. Categorical IQ does have an effect on DMR scores, with those in the <40 group having higher scores in Cognitive (<40 vs. ≥40: Estimate= -4.802, SE=1.356, p=0.00084) and total DMR (<40 vs. ≥40: Estimate =-5.895, SE=2.109, p=0.007). Categorical IQ stratified analyses were performed to take into account any possible specific IQ-dependent associations, however no significant associations between YoE and DMR Cognitive scores were observed in these stratified analysis.

On the other hand, although ApoE genotype is a known risk factor for dementia, it did not have a significant effect on DMR scores (results not shown).

Therefore, those in the <40 Categorical IQ group had higher DMR scores; however higher YoE were associated to lower DMR independently of Categorical IQ.

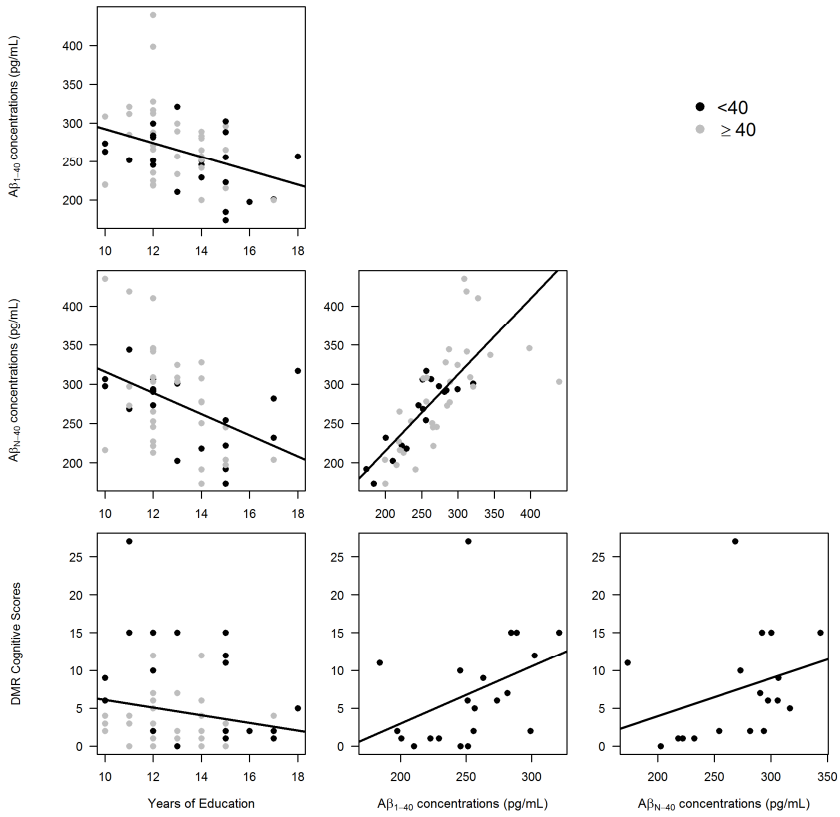

Figure 19: Correlation graphs for the studied variables. Graphs correspond from top to bottom and from left to right to: 1) Correlation between $A\beta_{1\text{-}40}$ and years of education, 2) Correlation between $A\beta_{N\text{-}40}$ and years of education, 3) Correlation Correlation between $A\beta_{1\text{-}40}$ and $A\beta_{N\text{-}40}$, 4) Correlation between DMR Cognitive scores and years of education, 5) Correlation between DMR Cognitive scores and $A\beta_{1\text{-}40}$, 6) Correlation between DMR Cognitive scores and $A\beta_{N\text{-}40}$.

5.3.5. Correlations of YoE with SVFT measures

No associations or correlations could be found between YoE and any of the SVFT measures used. However, in models adjusted for Categorical IQ, sex, age, and ApoE genotype, there were IQ-dependent differences in mean cluster size, with those in the <40 group producing smaller clusters (<40 vs. ≥40 Estimate=0.459, SE=0.222, p=0.04). No significant differences were observed for the total number of correct words (≥40 vs. <40 Estimate=1.987, SE=1.026, p=0.058). This implies that those in the <40 IQ group have lower semantic memory, which leads to a trend for a decreased word production.

Due to these differences observed for Categorical IQ, stratified analyses were performed. For the ≥40 group no correlation was found. On the other hand, for the <40 group correlations were observed between higher YoE and larger mean cluster size (rho=0.504, p=0.016, 95% CI=[0.152,0.734]). Therefore, individuals in the <40 group that have had longer educational periods have higher semantic memory.

Additionally, ApoE had an effect on the SVFT with ε2 carriers performing higher number of switches and producing more correct words than ε3 homozygotes. On the other hand, no differences could be observed between either of the groups and ε4 carriers **(Table 8)**. These differences show that ε2 carriers have higher mental flexibility, but that this higher flexibility also translates into a better performance in this task. Due to the sample size of both heterozygotes groups, no

statistical analyses were performed to further study the correlations between SVFT and YoE in each subgroup.

Table 8: ANCOVA results for the effect of ApoE genotype on the performance in the SVFT.

		ε3/ε3 vs. ε2/ε3	ε3/ε4 vs. ε2/ε3	ε3/ε3 vs. ε3/ε4
Number of switches	Estimate	-3.365	1.066	-2.299
	p	**0.011**	0.599	0.29
	95% CI	[-6.067,-0.662]	[-1.603,3.735]	[-5.976,1.378]
Total number of correct words	Estimate	-6.482	1.638	-4.843
	p	**0.0019**	0.61	0.12
	95% CI	[-10.779,-2.183]	[-2.595,5.873]	[-10.696,1.011]

Estimates show the differences between genotypes. CI: Confidence Interval; p: p-value.

5.3.6. Associations between YoE and $A\beta_{40}$ plasma concentrations

Models adjusted for YoE, sex, age, and categorical IQ show an association between YoE and $A\beta_{40}$ forms ($A\beta_{1\text{-}40}$: Estimate=-8.082, p=0.029, 95% CI=[-15.301,-0.861]; $A\beta_{N\text{-}40}$: Estimate=-13.461, p=0.003, 95% CI=[-22.347,-4.574]) (Fig. 1), with longer educational periods being associated to lower concentrations of both forms of $A\beta_{40}$.

None of the other variables, nor ApoE genotype, had an effect on $A\beta_{40}$ concentrations and therefore no stratified analyses were performed.

As previously described, there was an effect of Categorical IQ on DMR Cognitive Scores, with those in the <40 IQ having a higher mean score (≥40 vs <40: Estimate=-4.802, SE=1.356, p=0.00084).

Therefore, stratified analyses were performed. In the <40 group a significant direct correlation was found for $A\beta_{1-40}$ and Cognitive scores (rho=0.526, p=0.014, 95% CI=[0.130,0.800]), but not for $A\beta_{N-40}$ (rho=0.439, p=0.068, 95% CI=[-0.090,0.854]) (Fig. 1). In the ≥40 group no correlations were observed. Thus, lower DMR Cognitive scores in the <40 group correlated with decreased concentrations of $A\beta_{1-40}$.

5.4. Discussion

In this study we describe the direct associations between $A\beta_{40}$ and early signs of cognitive decline in individuals with IQ<40. Furthermore we analyzed the effect of longer educational periods (YoE) on dementia signs and biomarkers in a young population with DS. In particular, longer educational periods were associated to lower scores in DMR, better performance in the SVFT, and lower concentrations of Aβ40.

Our results suggest that $A\beta_{40}$ concentrations can be used as a biomarker for cognitive decline and that education can impact cognition and functionality before the dementia onset in DS.

5.4.1. $A\beta_{40}$ plasma concentrations

As it has been previously described (186, 187), no correlation between age and amyloid plasma concentrations could be observed.

The plasma concentrations of $A\beta_{1-40}$ we have obtained are comparable to the ones observed in studies with both older (186) and younger subjects with DS (77). The only study performed in a population of an

age closer to our study population reported lower concentrations than those of our study (78). Differences in the antibodies used in these measurements may explain these discrepancies. To our knowledge there are no other studies evaluating concentrations of $A\beta_{N-40}$ in young DS adults.

No effect of ApoE genotype on the peripheral biomarkers could be observed in our sample, possibly due to the low frequency of the ε2 and ε4 alleles, and to our sample size. However, this lack of effect has been previously described both in non-demented individuals with DS (186), and in young healthy adults (75).

5.4.2. Associations between $A\beta_{40}$ plasma concentrations and cognitive markers of cognitive decline

In a previous study, we already described the correlations between both DMR and SVFT with $A\beta_{42}$ (86), those individuals with higher plasmatic concentrations of $A\beta_{42}$ having worse performance in the SVFT and higher DMR scores. Because $A\beta_{40}$ plasma concentrations have been identified as a risk factor for the development of dementia in DS population (83, 84), so that older subjects with DS with dementia have significantly higher plasma concentrations of this peptide than non-demented subjects (84), we were interested in exploring the associations of this particular peptide and our previously established cognitive markers in young adults. Specially, as there is no previous data on correlations between $A\beta_{40}$ and cognitive decline signs in subjects with DS (79, 186).

In young adults, all the observed associations and correlations were IQ-dependent, and only significant in the <40 group. Similar to the results of previous study with $A\beta_{42}$, higher concentrations of $A\beta_{40}$ were correlated to higher DMR Cognitive Scores and to worse performance in the SVFT. In this latter case, both the full and the truncated form of $A\beta_{40}$ correlated to the number of words produced, however only the full form significantly correlated to the number of switches and therefore to lower mental flexibility and lower capacity to access the semantic memory.

5.4.3. Effects of YoE on cognitive markers of cognitive decline

One of the main characteristics of our population were the differences in both SVFT performance and DMR scores between categorical IQ groups. Individuals in the <40 group had higher DMR Cognitive and total scores, and worse performance in the SVFT. A previous study in older patients with DS showed differences in the pattern of appearance of dementia signs depending on the severity of the ID (188). Additionally, different cutoff values of DMR Cognitive scores for dementia diagnosis depending on IQ scores have been established, so that those with an IQ between 50 and 70 would be considered to have dementia with a score of 7 or higher, while those with an IQ between 25 and 50 should present a score of 20 or higher (189). According to this criterion, most of the individuals in our study and the mean of our group are not demented. However, one individual with an IQ of <40 and a score of 27, and another with an IQ of 70 and a score of 7 fulfill this criterion of dementia. The fact that

different cutoff values exist depending on IQ strengthens our observation for higher DMR scores in patients in the <40 group.

We also evaluated the potential impact of age and sex on cognitive decline, but no effect was found. In the case of age, this lack of effect is probably due to both the mean age and the age range of our sample, as all individuals were young.

Furthermore, the possible ApoE effect on cognition was also examined. Previous research in DS and cognitive decline showed disparities, with some works describing no effect of ApoE genotype in the progression to dementia (190), while other studies found a faster decline for ε4 carriers (191) as well as an association of this same allele with AD-DS (192). Our results, however, show no ApoE-dependent differences in DMR Scores, although this could be due to the sample size and frequencies of the alleles. Although the sample is different from that in **Chapter I**, we observe the same effect of the ApoE genotype on SVFT with ε2 carriers performing better. However, due to our sample size and genotype distribution, it is possible that larger samples render other results.

Independently of any of the possible confounding factors, we have an inverse association between YoE and DMR cognitive scores, with each extra year of education translating into a decrease of 0.779 in the score of the DMR cognitive scale. The scale of this impact is probably subclinical, but of enough interest to be taken into account. In the case of SVFT, YoE directly correlated to cluster size, indicating a more preserved semantic memory. However, this correlation was only significant in the IQ <40 group.

Both DMR and SVFT are considered to be indicators of cognitive decline in DS. In the case of DMR its use is widespread. The SVFT has been used as an early sign of cognitive decline in general population, and we have previously proposed it as a tool to evaluate cognitive decline in young population with DS (86). Therefore, putting our data in context, higher YoE correlates to lower early cognitive decline signs. The fact that the effect of YoE on SVFT is IQ dependent could be related to the differential IQ-dependent cognitive decline that we and others detected in population with DS (188).

These results would be in the same line to what has been described for DS, with subjects with higher level of schooling being associated to lower dementia symptoms (183), or elsewhere for the general population, with longer educational periods being protective against the development of AD (174-176).

5.4.4. Correlations and associations between YoE and $A\beta_{40}$ plasma concentrations

In the field of AD, intensive efforts have been put forward to obtaining a peripheral biomarker of disease development. Due to its role in the pathogenesis of AD, Aβ was considered to be the ideal candidate. However, it still has not proven to be adequate and its usefulness is extensively discussed (67). In DS, on the other hand, the use of this peripheral biomarker is more spread. One of the possible reasons for this is that individuals with DS have increased plasma Aβ concentrations throughout their lifetime due to the APP overexpression (77-81, 85). Additionally, plasma concentrations have shown to be predictive of cognitive decline and dementia conversion

in several studies (83-85), underscoring the usefulness of these biomarkers in this at risk population.

No data are available on the effects of YoE on amyloid concentrations in DS. In the general population the results are inconclusive (76, 180-182), adding more doubt about the usefulness of these biomarkers in AD. In our work, we observe that YoE are inversely associated to $A\beta_{40}$ concentrations, the effects being higher in the truncated form that in the full form, but significant in both cases. It is worth noting that, although the differences in concentrations are significant, the concentrations of $A\beta_{40}$ would still be higher than in age-matched euploid population. Nevertheless, considering the predictive effect on dementia conversion of these biomarkers, this described association between YoE and $A\beta_{40}$ could allow a better understanding of dementia risk in DS.

5.5. **Limitations**

One of the main limitations of this study is the small sample size and the ApoE allelic frequency that did not allow us to obtain enough carriers of the two minoritary alleles to fully assess the effect of ApoE in both cognition and peripheral biomarkers. Furthermore, this study is cross-sectional, and therefore it is not possible to assess whether the observed effects of YoE on plasma biomarkers and cognition are stable throughout time, or whether YoE are indeed protective against the AD-associated cognitive decline. To this end longitudinal and interventional studies should be performed in older and similar populations.

5.6. **Conclusions**

Amyloid peptides suitable biomarkers for cognitive decline in young individuals with DS, potentially allowing for an earlier detection of dementia risk in this population. Additionally, longer educational periods, that have been described as protective against dementia development, are associated to lower concentrations of plasmatic $A\beta_{40}$ as well as to fewer early cognitive markers of cognitive decline. Because of the intercorrelations of each of these variables with each other and to cognitive decline itself, it is possible that YoE could modify dementia risk in population with DS. Additionally, the fact that education can also modify peripheral amyloid concentrations could show the potential of this biomarker as biomarker of efficacy.

It is of particular interest that some of these correlations are only observed in the <40 IQ group pointing to either a different pattern in early cognitive decline or to an earlier onset of the early cognitive decline signs.

With this study we underline the importance of long educational periods as modulators of the early signs of cognitive decline. Furthermore, we reinforce the utility of both peripheral amyloid concentrations and SVFT to assess cognitive decline in young DS adults. Finally, we present a possible cognitive decline biomarker for young non-demented populations with DS.

6. CHAPTER IV: ONPCs as biomarkers

6.1. Introduction

One of the main difficulties when studying brain diseases or alterations in brain functions is our inability to obtain neuronal cells from live patients (112). This is particularly problematic when trying to assess the neuronal effects of a treatment in a clinical trial. The development of the induced pluripotent stem cells (iPSCs) technology has allowed obtaining neurons from live patients. However, this process requires the manipulation and differentiation of cells with a different fate and therefore it is lengthy and its efficiency is, sometimes, hampered (193). An alternative to iPSCs in neuropsychopharmacology studies are ONPCs that are of potential interest because they already have a neuronal fate and because many diseases of the central nervous system course with olfactory signs, proving that these cells are also affected (112).

There are two ways to obtain ONPCs from live patients, one is through biopsy of the nasal epithelium (194), and the other, developed by Dr. Benítez-King, is through the exfoliation of the olfactory neuroepithelium of the lower and medium turbinate (109). The advantages of the exfoliation method over the biopsy are that it is minimally invasive, painless, does not require a surgeon, and it requires less sample processing (109). This method has been already successfully used in schizophrenia and bipolar disorders (195), and in Williams syndrome (196)..

This chapter was developed during a research stay in Dr. Benítez-King laboratory with the objective to learn the sampling technique as well as the cell culture protocol and to assess whether the nasal exfoliate on itself could be used as a biomarker.

The adaptation of this technique to our laboratory has been lengthy. On one hand we first needed to validate the extraction method and the adequate interdental brushes to extract the sample, then we sought the approval of protocol by the Ethical Committee (CEIC), and finally we had to adapt and standardize the whole protocol to the conditions of our facilities. These delays have made it impossible to use this protocol in our study population, however it is being applied in other neuropsychopharmacology studies.

6.2. **Methods**

6.2.1. Sampling technique

Samples were obtained from young healthy volunteers during the morning. Patients were asked to clean their upper airways and a water nebulizer was used to humidify the nasal cavity. After the humidification, a sterile interdental brush was used to clean the lower part of the nasal cavity from one nostril, a second brush was used for further cleaning and sampling from the lower turbinate, finally a third brush was used to obtain sample from the middle turbinate. These two last brushes were placed inside Eppendorf tubes with 250µL of supplemented medium. The same procedure was performed for the other nostril. This protocol has been approved by the CEIC-PSMAR (2015/6979/I).

6.2.2. Cell culture

The Eppendorf tubes containing the brushes were kept in ice until sample processing. Such processing consisted on a first dislodgment of the sample by mechanical friction against a cut-off pipette tip, followed by a mechanical dissociation with micropipettes. Once the sample was dissociated, 50µL of the cell solution were seeded at an unknown concentration in 24-well plates, using different rows for the different nostril.

Cell medium was composed by DMEM/F-12 medium supplemented with 10% FBS, 2% glutamine, and 1% Antibiotic-Antimycotic. Medium was changed one week after seeding. Cells were maintained in the 24-well plate for one month or until confluence was reached. Once the cells were confluent, they were passaged. For the passage each well was first treated with 0.3mL 0.1% EDTA followed by 0.3mL 0.1% Trypsin to allow detachment for the plate. The reaction was neutralized with 1mL supplemented medium and cells were collected into 5mL Eppendorf tubes and centrifuged for 5 minutes at 1,000 rpm. After the centrifugation the medium was discarded and the cell pellet was resuspended in 1mL fresh supplemented medium. The cell solution was seeded in T25 flasks until confluency, the medium was changed every three days or as often as needed.

6.2.3. Nasal exfoliate staining protocol

In order to evaluate the utility of the nasal exfoliates as a primary biomarker, two experiments were conducted. For the first experiment exfoliates were seeded and fixed after 15-days in culture. In the second

experiment, a 14-day time course was performed fixing the cells every two days. Sampling and culture was performed as described, however cells were seeded on glass coverslips treated with Poly-L-lysine. Cells for the first experiment were fixed in 4% paraformaldehyde in day *in vitro* (DIV) 15, and stained for anti-nestin or for Anti-β-III tubulin with or without rhodamine-phalloidin staining, nuclei were stained with DAPI. Cells for the second experiment were fixed at DIV1, DIV2, DIV4, and DIV6. Double staining with anti-cytokeratin and phalloidin, and with anti-β-III tubulin and anti-nestin were performed. In both cases nuclei were stained with DAPI.

6.2.4. Serum deprivation protocol

Cells in passage 2 were seeded on poly-L-lysine treated coverslips, 60,000 cells were seeded per well with supplemented fresh medium. After four hours the medium was changed to serum free medium (DMEM-F12, 2% glutamine, 1% antibiotic-antimycotic). Cells were kept for 12 days, and fixed at DIV1, DIV4, and DIV8. Double staining with β-III tubulin and nestin was performed, with the nuclei being stained with DAPI.

6.3. **Results**

6.3.1. 15-day cell culture on coverslips

Nasal exfoliates were cultured in treated coverslips to evaluate the feasibility of using it as biomarker. Staining with anti-β-III tubulin and anti-nestin was performed to characterize the cells present in the sample. Almost all the cells expressed β-III tubulin, although some had higher expression levels than others **(Figure 20, panel A, arrow)**,

being such cells also larger, suggesting a later differentiation state. nestin expression was also ubiquitous; however the expression levels were similar across cells.

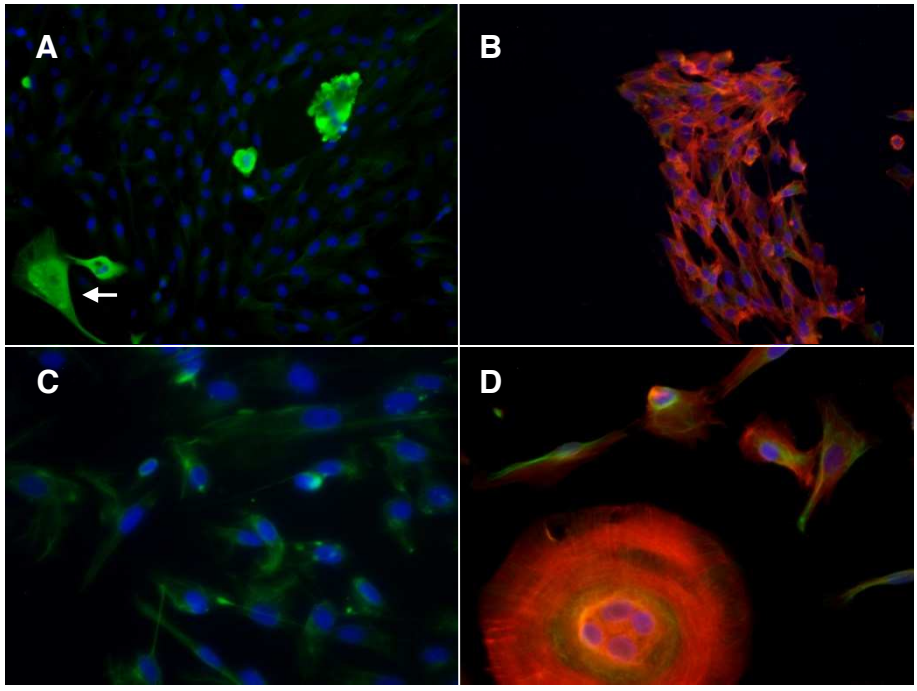

Figure 20: Immunohistochemical images of nasal exfoliate culture at DIV15. (A) Double staining with anti-β-III tubulin (green) and DAPI (blue) at 20x magnification. (B) Triple staining with anti-β-III tubulin (green), phalloidin (red), and DAPI (blue) at 20x magnification. (C) Double staining with anti-nestin (green) and DAPI (blue) at 40x magnification. (D) Triple staining with anti-nestin (green), phalloidin (red), and DAPI (blue) at 40x magnification.

6.3.2. Nasal exfoliates

After assessing the feasibility of using the nasal exfoliates as biomarkers, our interest was to determine which was the earliest time-point in which they could be employed. Staining with Anti-β-III tubulin and anti-nestin, and with anti-cytokeratin and phalloidin were performed at DIV1, DIV2, DIV4, and DIV6. On DIV1 few cells are

adhered to the coverslip, and most is debris produced during the sampling and processing **(Figures 21 and 22, panel A)**. On DIV2 small groups of cells can be observed, expression of both β-III tubulin and nestin is low **(Figure 21, panel B)**, while most cells seem to express cytokeratin **(Figure 22, panel B)**. On both DIV4 and DIV6, β-III tubulin and nestin expression seems to increase **(Figure 21, panels C and D)**, although there are cells negative for both antigens (nuclei signaled by arrows); in the case expression of cytokeratin seems to decrease as the culture advances **(Figure 22, panels C and D)**.

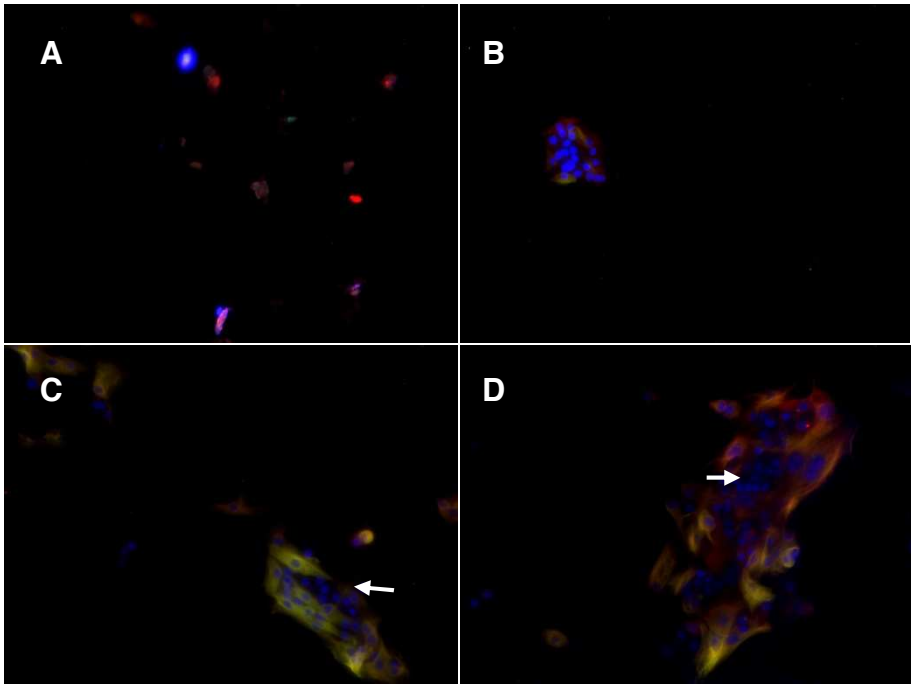

Figure 21: 20x magnification images of triple staining with Anti-β-III tubulin (green), Anti-Nestin (red), and DAPI (blue). (A) Sample after 24 hours (DIV1). (B) Sample after 48 hours (DIV2). (C) Sample at DIV4. (D) Sample at DIV6.

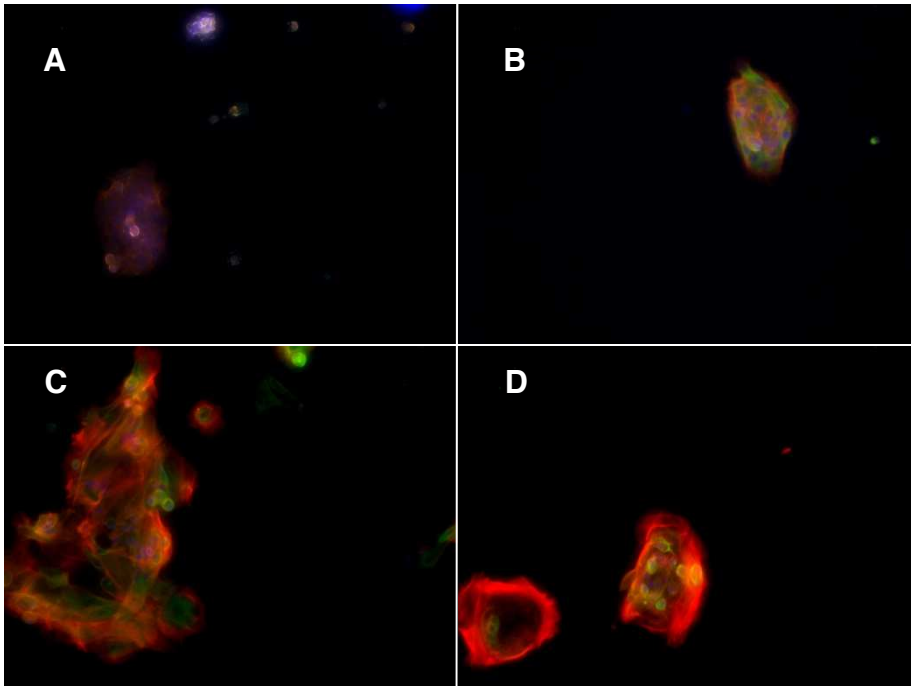

Figure 22: 20x magnification images of triple staining with anti-cytokeratin (green), phalloidin (red), and DAPI (blue). (A) Sample after 24 hours (DIV1). (B) Sample after 48 hours (DIV2). (C) Sample at DIV4. (D) Sample at DIV6.

6.3.3. Serum deprivation experiment

In order to assess differentiation, cells were cultured in serum free medium. Staining with anti-β-III tubulin and anti-nestin was performed. After 24-hours (DIV1) cells had attached to the coverslip and had an elongated morphology **(Figure 23, panels A and B)** that was maintained and increased on DIV4 and DIV8 **(Figure 23, panels from C to F)**. At all time-points cells were positive for both antigens.

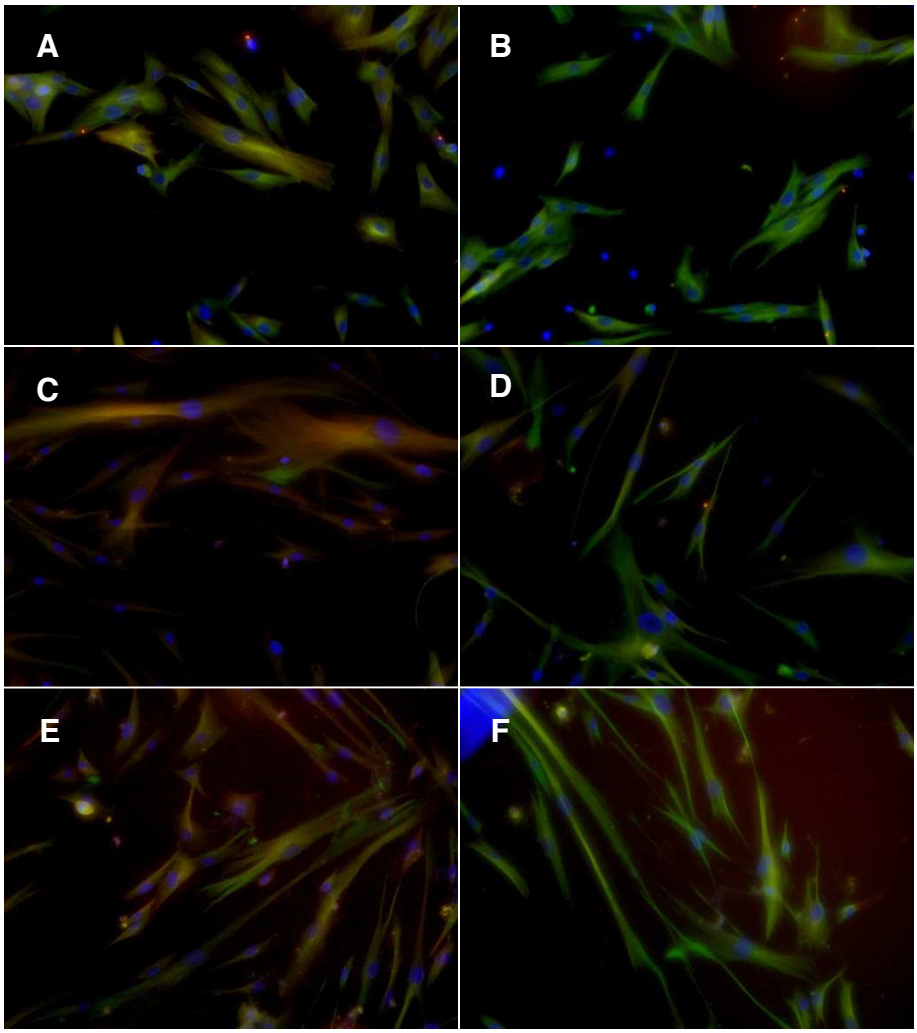

Figure 23: 20x magnification images of triple staining with anti-β-III tubulin (red), anti-nestin (green), and DAPI (blue). (A and B) Serum deprived cells at DIV1. (C and D) Serum deprived cells at DIV4. (E and F) Serum deprived cells at DIV8.

6.4. Discussion

We have developed and validated a methodology for the procurement of ONPCs and nasal exfoliates and their further differentiation. The usefulness of these cells as potential source of biomarkers in clinical trials.is pending of further evaluation.

6.4.1. Nasal exfoliate as a source of neuronal biomarkers

Previous research using ONPCs obtained from nasal exfoliates had focused on exfoliates as a source of ONPCs rather than a biomarker on its own (109). In this study we have proven that nasal exfoliates cultured for 15 days on Poly-L-lysine coverslips have cells expressing both nestin and β-III tubulin. β-III tubulin has been described to be expressed mainly in immature and mature olfactory sensory neurons, while nestin is usually considered a neural progenitor marker, although its expression in ONPCs has been discussed (112). Our cells after 15 days in culture expressed both nestin and β-III tubulin. However, due to the low availability of sample in this experiment, we could not perform double staining experiments and we could not identify if cells were positive for both markers. Nevertheless, we could observe that some cells had a stronger β-III tubulin staining than others while at the same time being larger. Both of these signs suggest that those cells were at a later differentiation state, although their presence was anecdotic. Regardless, the generalized expression of β-III tubulin seems to indicate that on DIV15 most of the cells are indeed of neuronal lineage. Furthermore, the expression of nestin would imply that they are in fact progenitors.

In order to pinpoint the earliest time point in which those cells could be used for biomarker studies, a time-course experiment of the nasal exfoliate was performed. Cells were fixed at DIV1, DIV2, DIV4, and DIV8. Not surprisingly, at DIV1 there were not enough cells attached to the coverslips and only debris could be observed. Whether it was because cells had not attached or because the attachment was too weak, remains to be determined. Starting from DIV2 there were cells attached to the coverslip. Double immunostaining with β-III tubulin and nestin shows that at DIV2 some of the cells express nestin, with few of them expressing both. Additionally, DAPI nuclei staining showed how there were some cells that were not positive for any of the markers and are possibly cells that are not of neuronal lineage, although that cannot be discarded. At this same DIV, cytokeratin staining was predominant for all the cells, showing the epithelial character of these cells. Both at DIV4 and DIV8 cells expressed both β-III tubulin and nestin, highlighting the presence of cells of neuronal lineage in the sample. As with the DIV2 staining, there were cells negative for both markers, often in the same growth regions as the neuronal lineage cells, on DIV8 the nuclei of these cells look smaller than the neighboring positive cells. On the same period, cells were still positive for cytokeratin, although the staining seemed to diminish as the culture advanced. Based on these observations our culture was formed by both neuronal and non-neuronal cells, however we cannot assess whether any of the cells expressed both neuronal and epithelial markers. This time course experiment allowed us to determine that from DIV4 these cells could already be used to identify potential biomarkers.

6.4.2. Serum deprivation effect on ONPCs

The final experiment of this chapter was done to examine the effect of serum deprivation on passage 2 cells. Numerous differentiation protocols have been used on cells obtained from the nasal epithelium, and serum deprivation is one of them. Serum deprivation has proven to increase expression of β-III tubulin (112). Thus, cells were deprived of serum for 6 days and fixed at DIV1, DIV4, and DIV8. At all time-points they expressed both β-III tubulin and nestin, although their morphology changed considerably with their nuclei being larger and the cells being more elongated. However, we could not fully assess the degree of differentiation of these cells using only these two markers. Nevertheless, we have proven that cells obtained through nasal exfoliation are able, under serum deprivation conditions, to acquire morphology consistent with later stages of neuronal differentiation.

6.5. **Limitations**

Despite being a fast and generally reliable method, it still has several limitations. The efficacy of the method greatly depends on the ability to obtain enough precursor cells from the volunteer, which cannot be assessed until the cells have been in culture for some days and it is difficult to implement due to interindividual variability. Additionally, due to the nature of the source tissue, contaminations happen frequently, despite the use of antibiotics and antimycotics. Special care needs to be taken when handling primary cultures and usually separated cultures for each volunteer are kept in order to preserve the original culture. In some cases the cell culture does not succeed, having cells die before first passage, or immediately after. We are

currently working on improving sampling and culture method. So far we have introduced the use of local anesthesia to increase the comfort of the volunteers, and we have implemented measures to avoid contamination and cross-contamination of the samples.

6.6. **Conclusions**

Nasal exfoliate cultures provide cells from neuronal lineage at DIV15 and can be used as biomarker source as soon as DIV4. Furthermore, serum deprivation has shown to affect the morphology of the ONPCs and this could be used as differentiation protocol. In conclusion, we demonstrated that both nasal exfoliates and ONPCs are suitable sources for biomarker studies in clinical trials.

7. GENERAL DISCUSSION

There is limited knowledge on the associations between peripheral biomarkers and cognition in individuals with ID. This knowledge would be of vital importance because specific biomarkers have been identified as risk factors for cognitive decline in the general population, and such factors can be modified before the decline appears. These biomarkers could also be used as treatment efficacy markers in the framework of clinical trials. With this work we describe the relationship between cognitive and functional variables and peripheral biomarkers in DS. Additionally, we explore the usefulness of ONPCs as a proxy of neural tissue to explore them as biomarkers of treatment efficacy and as cellular disease model.

In our first study **(Chapter I)**, we examine the associations between EF and lipid profile, both at baseline and after a one-year treatment with EGCG and cognitive training. An altered lipid profile has long been identified as a risk factor for cognitive decline in the general population (98-103, 120, 121), and has been associated to specific cognitive domains throughout lifetime (98-100, 104-106). Furthermore, catechins of green tea and specifically EGCG have proven to modify the peripheral lipid concentrations in several different conditions (119, 128-133) and in our studies in population with DS (10, 13).

The results of the clinical trial also showed the capacity of this therapeutic approach to improve the performance in tasks related to EF (13). Due to these effects of the treatment we evaluated the associations between lipid profile and EF. One of our main findings

was that these associations were sex dependent. This fact can be explained because females have higher concentrations of HDLc and better performance in planning tasks. Our results also showed that a visuo-spatial attention task is associated to a better lipid profile in males, particularly in those displaying higher HDLc and lower TC concentrations. This same lipid profile is associated to better planning skills in females. All these observations translate into associations in functionality, as measured by the ABAS-II, with higher HDLc concentrations being related to better adaptive behavior (Results are summarized in **Figures 24 and 25**). Specific relations with other components of the lipid profile could be observed. On one hand, oxLDL concentrations were associated to better verbal attention, in males, and better functionality, regardless of sex. Such effect has never been described before and it could be related to the association of oxLDL with fT_4 concentrations, although thyroid hormones had no influence on the association between oxLDL and cognition. Also, higher concentrations of TC were associated to better scores in the Self-Care subscale of ABAS-II. In this case, the scores of this subscale were higher in the MeDi compliant group and this could explain the association between TC and functionality, even if there was no association between TC and MeDi compliance. Potential modifiers of the lipid profile such as MeDi and ApoE were analyzed for their effects on both lipid profile and cognition. No effect of any of them could be observed in lipid profile which could be due to our limited sample size. Nevertheless, they did have an influence on cognition; in particular ε2 carriers of the ApoE genotype had a better performance in the SVFT (which can also be seen in **Chapter III**) and MeDi

compliance was associated to better adaptive behavior and fewer dementia signs (also in **Chapter II**)**(Figures 25 and 26)**.

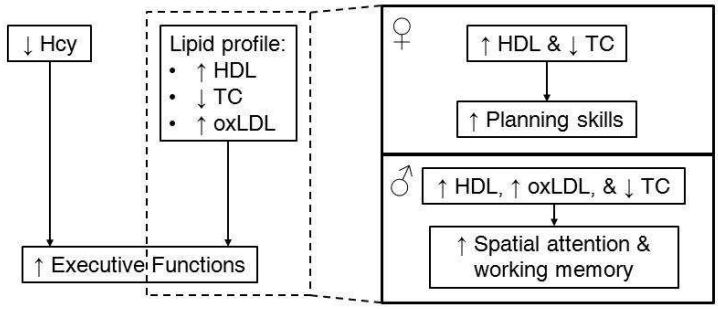

Figure 24: Schematic representation of the main findings with regards to peripheral biomarkers and executive functions. The results for the lipid profile are further detailed in the two boxes in the left (top: females, bottom: males).

When analyzing the results from the longitudinal part of this study we observed that EGCG treatment is able to modify the lipid profile in a sex-dependent manner, and to improve a specific functional domain in a sex-independent manner. Longitudinal associations in the placebo group show that changes in lipid profile were also associated with changes in planning skills and in functionality, although these effects were only observed in females. The treatment with EGCG and CT annulled some of these associations while giving rise to new ones in which decreases in TC and LDLc concentrations were related to increases in ABAS-II Functional Academics.

Taking together these results, the lipid profile shows a clear association to cognition meaning that it has the potential to become a biomarker of cognitive function also in DS. Furthermore, and especially due to its association with cognitive changes in the EGCG and cognitive training-treated group, it could also be used as a

biomarker for the cognitive changes caused by the treatment. However, all these associations tend to be sex-dependent.

Following the results from this first study, we explored the associations between EF and Hcy in **Chapter II**. Individuals with DS have lower concentrations of Hcy than age-matched controls (159), and we have successfully used Hcy as efficacy biomarker in our clinical trials with EGCG (10, 13), where increases of Hcy showed a possible reduction of DYRK1A activity. However, previous research in DS, showed that Hcy inversely correlated with IQ (160), and in general population Hcy concentrations are known to be associated to worse EF (27, 29, 32, 35). Consequently, we were interested in understanding the basal associations between EF and Hcy concentrations in our population. Furthermore, because of the effect of EGCG and cognitive training on both cognition and Hcy concentrations, we wanted to assess whether these changes were associated in a longitudinal study.

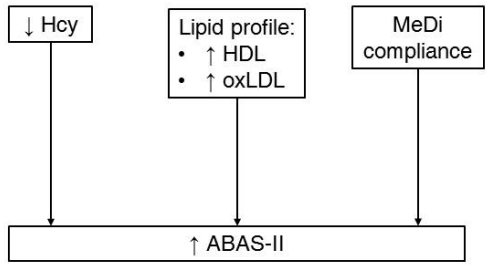

Figure 25: Schematic representation of the main findings with regards to peripheral biomarkers, other modifiers, and functionality.

Our results show that higher concentrations of Hcy are associated to worse planning skills and worse functionality including Total ABAS-II

score **(Figures 24 and 25)**. As discussed in the chapter, we were not expecting such results due to the known secondary effect of DYRK1A activity on Hcy concentrations, therefore potential modifiers of Hcy concentrations were analyzed. Diet and genetic polymorphisms of the One Carbon Metabolism are known modulators of Hcy concentrations. However, we could not observe any effect of these confounders in our sample. In the cognitive function we observe an effect of the MTR polymorphism on both IQ raw scores and functionality. Additionally, and as in **Chapter I**, MeDi was associated to functionality and cognitive decline scores **(Figure 26)**.

In this study, there were fewer longitudinal associations than for Chapter II, and only one of them was found for the EGCG and cognitive training group. This lack of associations compared to basal conditions suggests that, although Hcy is a good biomarker of efficacy, it is not of cognitive change derived from EGCG treatment.

The first two chapters have shown an association between MeDi compliance and early signs of dementia. In **Chapter III** we analyzed the association between amyloid peripheral biomarkers and early signs of cognitive decline, and the influence of years of education. In the general population, it has long been described that longer educational periods and higher educational attainments are associated to lower risk of dementia (174-176). The effects of education in peripheral amyloidosis biomarkers are less clear (76, 180-182) and the utility of such biomarkers is still under consideration (67, 71). In individuals with DS, plasma amyloid concentrations have proven to be predictive of AD risk (82-84). In our population, higher $A\beta_{40}$ concentrations were associated to higher early dementia signs, while longer

educational periods were to lower scores in the DMR and better performances in the SVFT, and lower peripheral amyloid concentrations **(Figure 26)**. Similarly to what we did for **Chapter I**, ApoE role was investigated for these variables, in this case, as previously, ApoE genotype only had an effect on the SVFT performance.

This last chapter, allows us to consider the use of plasma amyloid concentrations as cognitive decline biomarkers, and, due to the effect of YoE on them, to suggest that they could be used as efficacy biomarkers.

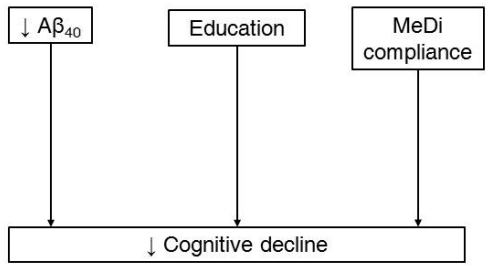

Figure 26: Schematic representation of the main findings with regards to peripheral biomarkers, other modifiers, and cognitive decline.

With the results of these three studies, we observe that the associations between peripheral biomarkers and cognition in individuals with DS are similar to those already observed in the general population. In brief, the lipid profile, and in particular HDLc concentrations, is associated to executive functions, as are Hcy concentrations. Furthermore, peripheral amyloid concentrations correlate to early dementia signs, underlining their usefulness to assess dementia risk in DS. Nevertheless, we found specific associations

between peripheral biomarkers and cognition such as the one between oxLDL and functionality that had never been observed in the general population; which could be related to both thyroid hormones and oxidation.

Such associations between peripheral biomarkers and cognition allow us to demonstrate the potential of these biomarkers as markers of cognition, cognitive change, and /or efficacy. Furthermore, we have proven that specific interventions can affect biomarkers, cognition, and/or their association.

However, the results of these first three studies rely on peripheral biomarkers concentrations which are, sometimes, a poor readout of what is really happening in the brain. In order to address this problem we have explored new sources of biomarkers in **Chapter IV**. ONPCs have been used to study specific neurological diseases and as a source of neuronal precursors. We evaluated the possibility of using them as biomarkers by determining the availability of neuronal lineage cells on the cell culture as soon as DIV4. Furthermore, we have also assessed the potential of these cells to differentiate in serum free media, although the results were not conclusive. Regardless, ONPCs would provide a novel and accurate biomarker to analyze the effects that a pharmacological treatment is having on neurons in an easy and non-invasive way.

8. LIMITATIONS AND FUTURE DIRECTIONS

The main limitations of this thesis are derived from the characteristics of the clinical trial in which it is nested. On one hand, we are limited by the number of patients that were recruited, although it is the largest reported. For this reason some genetic alleles were insufficiently represented to fully understand the modulating effects they could be having on our variables. Furthermore, the fact that we could not obtain the MeDi compliance questionnaire for all individuals supposed that interactions between genetic polymorphisms and dietary patterns on any other variables could not be calculated. One last consequence of the reduced number of individuals was that we could not adjust our alpha risk for multiple comparisons, for this reason we considered these studies to be exploratory. Additionally, **Chapter III** has focused only on the transversal associations between amyloidosis biomarkers and cognition, when a longitudinal analysis could have helped to better understand the effects of the biomarkers on cognition, or the effects of external factors on cognition. It is worth noting that for the amyloid biomarkers, longitudinal concentrations were obtained, however they were dismissed due to variability between assays. Furthermore, in none of the cases have we explored the effect of basal concentrations of biomarkers on the changes of cognition, which would be useful as it would give us a predictive biomarker. In the case of **Chapter IV** our limitations were mostly due to technical difficulties with the culture technique and the specificity of the antibodies in Mexico City and with setting up the method in our laboratory. It meant that the time-course experiments and specific staining to further characterize the cells could not be performed as planned.

The results of this thesis open an avenue for further research. On one hand, further studies are needed to fully understand the effects of our biomarkers in individuals with DS, not only on EF, but also on dementia risk. To this end a longitudinal study would be required following individuals for most of their lifespan and assessing cognition and cognitive decline. Following the same line, a study to determine whether treatments that modify peripheral lipid concentrations also have an effect on cognition could provide with new enhancing treatments in DS. Additionally, we have observed effects of education on early signs of dementia and peripheral amyloidosis markers and it is possible that cognitive stimulation programs could have a similar effect. Related to the results of the clinical trial, studies in both pediatric and older population are needed to assess the early effects of an intervention in cognition in children with DS and the possible protective effect of EGCG on cognitive decline. Regarding the possibility of using the ONPCs as biomarkers, there is a need to fully optimize the method while assessing the rate of cells obtained from patients with DS. Once these two aspects have been addressed, ONPCs could start to be analyzed on the search of new biomarkers in a neural surrogate tissue.

9. CONCLUSIONS

I. In Down syndrome young adults the lipid profile is associated with executive function being higher concentrations of HDLc and lower concentrations of total cholesterol associated to better executive function.

   a. These associations are different in males and females, specifically regarding executive functions, while adaptive behavior showed no sex-dependent differences.

   b. Neither ApoE nor compliance with a Mediterranean diet affect lipid profile probably due to the reduced sample size. Nevertheless, both factors modulate cognitive performance of individuals. While ApoE genotype of subjects affects verbal fluency of subjects (Semantic Verbal Fluency Task), compliance with a Mediterranean diet has a protective effect on early signs of dementia and cognitive decline and improves adaptive behavior in this population.

II. EGCG treatment modifies the concentrations of total cholesterol, LDLc, oxLDL, and triglycerides in a sex-dependent manner. Additionally it improves the scores of the Functional Academics ABAS-II subscale in a sex-dependent manner. Lipid profile can also be used as a cognitive-change biomarker.

III. Higher homocysteine concentrations are a good cognition biomarker since they are associated to worse executive function independent of gender, one carbon genetic polymorphisms, and Mediterranean diet compliance. However, EGCG treatment increases homocysteine concentrations while improving specific

functional domains, suggesting additional modulating factor in regulatory mechanisms.

IV. Amyloid biomarkers are associated to early cognitive decline being both reduced by longer educational periods. This would imply that education has a potential protective effect against cognitive decline.

V. ONPCs express neuronal markers and prove the potential of these cells as a source for biomarkers.

VI. In conclusion, our study has identified biomarkers of cognitive performance in young Down syndrome individuals. Some of them show a clear sexual dimorphism that is also detected in the cognitive profile of the Down syndrome population. Some are useful as cognitive markers in baseline condition but may not reflect the cognitive changes induced by therapeutic interventions.

References

1. Patterson D. Molecular genetic analysis of Down syndrome. Hum Genet. 2009 Jul;126(1):195-214.
2. Chen CC, Ringenbach SD, Crews D, Kulinna PH, Amazeen EL. The association between a single bout of moderate physical activity and executive function in young adults with Down syndrome: a preliminary study. J Intellect Disabil Res. 2015 Jul;59(7):589-98.
3. Diamond A. Executive functions. Annu Rev Psychol. 2013;64:135-68.
4. Grieco J, Pulsifer M, Seligsohn K, Skotko B, Schwartz A. Down syndrome: Cognitive and behavioral functioning across the lifespan. Am J Med Genet C Semin Med Genet. 2015 Jun;169(2):135-49.
5. Altafaj X, Dierssen M, Baamonde C, Marti E, Visa J, Guimera J, et al. Neurodevelopmental delay, motor abnormalities and cognitive deficits in transgenic mice overexpressing Dyrk1A (minibrain), a murine model of Down's syndrome. Hum Mol Genet. 2001 Sep 1;10(18):1915-23.
6. Dierssen M, de Lagran MM. DYRK1A (dual-specificity tyrosine-phosphorylated and -regulated kinase 1A): a gene with dosage effect during development and neurogenesis. ScientificWorldJournal. 2006;6:1911-22.
7. Prasher VP, Farrer MJ, Kessling AM, Fisher EM, West RJ, Barber PC, et al. Molecular mapping of Alzheimer-type dementia in Down's syndrome. Ann Neurol. 1998 Mar;43(3):380-3.
8. Ryoo SR, Cho HJ, Lee HW, Jeong HK, Radnaabazar C, Kim YS, et al. Dual-specificity tyrosine(Y)-phosphorylation regulated kinase 1A-mediated phosphorylation of amyloid precursor protein: evidence for a functional link between Down syndrome and Alzheimer's disease. J Neurochem. 2008 Mar;104(5):1333-44.
9. Wegiel J, Gong CX, Hwang YW. The role of DYRK1A in neurodegenerative diseases. FEBS J. 2011 Jan;278(2):236-45.
10. De la Torre R, De Sola S, Pons M, Duchon A, de Lagran MM, Farre M, et al. Epigallocatechin-3-gallate, a DYRK1A inhibitor, rescues cognitive deficits in Down syndrome mouse models and in humans. Mol Nutr Food Res. 2014 Feb;58(2):278-88.
11. Guedj F, Sebrie C, Rivals I, Ledru A, Paly E, Bizot JC, et al. Green tea polyphenols rescue of brain defects induced by overexpression of DYRK1A. PLoS One. 2009;4(2):e4606.

12. Sang S, Lambert JD, Ho CT, Yang CS. The chemistry and biotransformation of tea constituents. Pharmacol Res. 2011 Aug;64(2):87-99.
13. de la Torre R, de Sola S, Hernandez G, Farré M, Pujol J, Rodriguez J, et al. Safety and efficacy of cognitive training plus epigallocatechin-3-gallate in young adults with Down's syndrome (TESDAD): a double-blind, randomised, placebo-controlled, phase 2 trial. The Lancet Neurology. 2016;15(8):801-10.
14. Strimbu K, Tavel JA. What are biomarkers? Curr Opin HIV AIDS. 2010 Nov;5(6):463-6.
15. Delabar JM, Theophile D, Rahmani Z, Chettouh Z, Blouin JL, Prieur M, et al. Molecular mapping of twenty-four features of Down syndrome on chromosome 21. Eur J Hum Genet. 1993;1(2):114-24.
16. Dierssen M. Down syndrome: the brain in trisomic mode. Nat Rev Neurosci. 2012 Dec;13(12):844-58.
17. Lott IT, Dierssen M. Cognitive deficits and associated neurological complications in individuals with Down's syndrome. Lancet Neurol. 2010 Jun;9(6):623-33.
18. Kittler PM, Krinsky-McHale SJ, Devenny DA. Dual-task processing as a measure of executive function: a comparison between adults with Williams and Down syndromes. Am J Ment Retard. 2008 Mar;113(2):117-32.
19. Rowe J, Lavender A, Turk V. Cognitive executive function in Down's syndrome. Br J Clin Psychol. 2006 Mar;45(Pt 1):5-17.
20. Lee NR, Anand P, Will E, Adeyemi EI, Clasen LS, Blumenthal JD, et al. Everyday executive functions in Down syndrome from early childhood to young adulthood: evidence for both unique and shared characteristics compared to youth with sex chromosome trisomy (XXX and XXY). Front Behav Neurosci. 2015;9:264.
21. Maatta T, Tervo-Maatta T, Taanila A, Kaski M, Iivanainen M. Mental health, behaviour and intellectual abilities of people with Down syndrome. Downs Syndr Res Pract. 2006 Aug;11(1):37-43.
22. Dekker AD, Strydom A, Coppus AM, Nizetic D, Vermeiren Y, Naude PJ, et al. Behavioural and psychological symptoms of dementia in Down syndrome: Early indicators of clinical Alzheimer's disease? Cortex. 2015 Dec;73:36-61.
23. Weksler ME, Szabo P, Relkin NR, Reidenberg MM, Weksler BB, Coppus AM. Alzheimer's disease and Down's syndrome: treating two paths to dementia. Autoimmun Rev. 2013 Apr;12(6):670-3.
24. Head E, Powell D, Gold BT, Schmitt FA. Alzheimer's Disease in Down Syndrome. Eur J Neurodegener Dis. 2012 Dec;1(3):353-64.

25. Brosnan JT, Brosnan ME. The sulfur-containing amino acids: an overview. J Nutr. 2006 Jun;136(6 Suppl):1636S-40S.
26. Seshadri S, Beiser A, Selhub J, Jacques PF, Rosenberg IH, D'Agostino RB, et al. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N Engl J Med. 2002 Feb 14;346(7):476-83.
27. West RK, Beeri MS, Schmeidler J, Mitchell DB, Carlisle KR, Angelo G, et al. Homocysteine and cognitive function in very elderly nondemented subjects. Am J Geriatr Psychiatry. 2011 Jul;19(7):673-7.
28. Sedille-Mostafaie N, Zehetmayer S, Krampla W, Krugluger W, Fischer P. Influence of vascular risk factors on executive function among an age-homogeneous elderly cohort. J Neural Transm (Vienna). 2015 Sep;122(9):1323-8.
29. Ford AH, Flicker L, Singh U, Hirani V, Almeida OP. Homocysteine, depression and cognitive function in older adults. J Affect Disord. 2013 Nov;151(2):646-51.
30. Beydoun MA, Beydoun HA, Gamaldo AA, Teel A, Zonderman AB, Wang Y. Epidemiologic studies of modifiable factors associated with cognition and dementia: systematic review and meta-analysis. BMC Public Health. 2014;14:643.
31. Allam M, Fahmy E, Elatti SA, Amer H, Abo-Krysha N, El-Sawy E. Association between total plasma homocysteine level and cognitive functions in elderly Egyptian subjects. J Neurol Sci. 2013 Sep 15;332(1-2):86-91.
32. Sala I, Belen Sanchez-Saudinos M, Molina-Porcel L, Lazaro E, Gich I, Clarimon J, et al. Homocysteine and cognitive impairment. Relation with diagnosis and neuropsychological performance. Dement Geriatr Cogn Disord. 2008;26(6):506-12.
33. Ganguli M, Fu B, Snitz BE, Unverzagt FW, Loewenstein DA, Hughes TF, et al. Vascular risk factors and cognitive decline in a population sample. Alzheimer Dis Assoc Disord. 2014 Jan-Mar;28(1):9-15.
34. Sachdev P. Homocysteine, cerebrovascular disease and brain atrophy. J Neurol Sci. 2004 Nov 15;226(1-2):25-9.
35. Raszewski G, Loroch M, Owoc A, Lukawski K, Filip R, Bojar I. Homocysteine and cognitive disorders of postmenopausal women measured by a battery of computer tests--central nervous system vital signs. Arch Womens Ment Health. 2015 Aug;18(4):623-30.
36. Pogribna M, Melnyk S, Pogribny I, Chango A, Yi P, James SJ. Homocysteine metabolism in children with Down syndrome: in vitro modulation. Am J Hum Genet. 2001 Jul;69(1):88-95.

37. Chadefaux B, Ceballos I, Hamet M, Coude M, Poissonnier M, Kamoun P, et al. Is absence of atheroma in Down syndrome due to decreased homocysteine levels? Lancet. 1988 Sep 24;2(8613):741.
38. Chadefaux B, Rethore MO, Raoul O, Ceballos I, Poissonnier M, Gilgenkranz S, et al. Cystathionine beta synthase: gene dosage effect in trisomy 21. Biochem Biophys Res Commun. 1985 Apr 16;128(1):40-4.
39. Ji J, Lee H, Argiropoulos B, Dorrani N, Mann J, Martinez-Agosto JA, et al. DYRK1A haploinsufficiency causes a new recognizable syndrome with microcephaly, intellectual disability, speech impairment, and distinct facies. Eur J Hum Genet. 2015 Nov;23(11):1473-81.
40. Tejedor FJ, Hammerle B. MNB/DYRK1A as a multiple regulator of neuronal development. FEBS J. 2011 Jan;278(2):223-35.
41. Dowjat WK, Adayev T, Kuchna I, Nowicki K, Palminiello S, Hwang YW, et al. Trisomy-driven overexpression of DYRK1A kinase in the brain of subjects with Down syndrome. Neurosci Lett. 2007 Feb 8;413(1):77-81.
42. Martinez de Lagran M, Benavides-Piccione R, Ballesteros-Yanez I, Calvo M, Morales M, Fillat C, et al. Dyrk1A influences neuronal morphogenesis through regulation of cytoskeletal dynamics in mammalian cortical neurons. Cereb Cortex. 2012 Dec;22(12):2867-77.
43. Park J, Chung KC. New Perspectives of Dyrk1A Role in Neurogenesis and Neuropathologic Features of Down Syndrome. Exp Neurobiol. 2013 Dec;22(4):244-8.
44. Ortiz-Abalia J, Sahun I, Altafaj X, Andreu N, Estivill X, Dierssen M, et al. Targeting Dyrk1A with AAVshRNA attenuates motor alterations in TgDyrk1A, a mouse model of Down syndrome. Am J Hum Genet. 2008 Oct;83(4):479-88.
45. Altafaj X, Martin ED, Ortiz-Abalia J, Valderrama A, Lao-Peregrin C, Dierssen M, et al. Normalization of Dyrk1A expression by AAV2/1-shDyrk1A attenuates hippocampal-dependent defects in the Ts65Dn mouse model of Down syndrome. Neurobiol Dis. 2013 Apr;52:117-27.
46. Liu F, Liang Z, Wegiel J, Hwang YW, Iqbal K, Grundke-Iqbal I, et al. Overexpression of Dyrk1A contributes to neurofibrillary degeneration in Down syndrome. FASEB J. 2008 Sep;22(9):3224-33.
47. Ryoo SR, Jeong HK, Radnaabazar C, Yoo JJ, Cho HJ, Lee HW, et al. DYRK1A-mediated hyperphosphorylation of Tau. A

functional link between Down syndrome and Alzheimer disease. J Biol Chem. 2007 Nov 30;282(48):34850-7.
48. Ferrer I, Barrachina M, Puig B, Martinez de Lagran M, Marti E, Avila J, et al. Constitutive Dyrk1A is abnormally expressed in Alzheimer disease, Down syndrome, Pick disease, and related transgenic models. Neurobiol Dis. 2005 Nov;20(2):392-400.
49. Noll C, Planque C, Ripoll C, Guedj F, Diez A, Ducros V, et al. DYRK1A, a novel determinant of the methionine-homocysteine cycle in different mouse models overexpressing this Down-syndrome-associated kinase. PLoS One. 2009;4(10):e7540.
50. Noll C, Tlili A, Ripoll C, Mallet L, Paul JL, Delabar JM, et al. Dyrk1a activates antioxidant NQO1 expression through an ERK1/2-Nrf2 dependent mechanism. Mol Genet Metab. 2011 Dec 2.
51. Naik S, Joglekar C, Bhat D, Lubree H, Rege S, Raut K, et al. Marked gender difference in plasma total homocysteine concentrations in indian adults with low vitamin B(1)(2). Int J Vitam Nutr Res. 2011 Sep;81(5):306-16.
52. Norlund L, Grubb A, Fex G, Leksell H, Nilsson JE, Schenck H, et al. The increase of plasma homocysteine concentrations with age is partly due to the deterioration of renal function as determined by plasma cystatin C. Clin Chem Lab Med. 1998 Mar;36(3):175-8.
53. Stanislawska-Sachadyn A, Woodside JV, Brown KS, Young IS, Murray L, McNulty H, et al. Evidence for sex differences in the determinants of homocysteine concentrations. Mol Genet Metab. 2008 Apr;93(4):355-62.
54. Fukagawa NK, Martin JM, Wurthmann A, Prue AH, Ebenstein D, O'Rourke B. Sex-related differences in methionine metabolism and plasma homocysteine concentrations. Am J Clin Nutr. 2000 Jul;72(1):22-9.
55. Strassburg A, Krems C, Luhrmann PM, Hartmann B, Neuhauser-Berthold M. Effect of age on plasma homocysteine concentrations in young and elderly subjects considering serum vitamin concentrations and different lifestyle factors. Int J Vitam Nutr Res. 2004 Mar;74(2):129-36.
56. Biselli JM, Zampieri BL, Goloni-Bertollo EM, Haddad R, Fonseca MF, Eberlin MN, et al. Genetic polymorphisms modulate the folate metabolism of Brazilian individuals with Down syndrome. Mol Biol Rep. 2012 Oct;39(10):9277-84.
57. Dedoussis GV, Panagiotakos DB, Chrysohoou C, Pitsavos C, Zampelas A, Choumerianou D, et al. Effect of interaction between adherence to a Mediterranean diet and the methylenetetrahydrofolate

reductase 677C-->T mutation on homocysteine concentrations in healthy adults: the ATTICA Study. Am J Clin Nutr. 2004 Oct;80(4):849-54.
58. Fillon-Emery N, Chango A, Mircher C, Barbe F, Blehaut H, Herbeth B, et al. Homocysteine concentrations in adults with trisomy 21: effect of B vitamins and genetic polymorphisms. Am J Clin Nutr. 2004 Dec;80(6):1551-7.
59. Tunbridge EM, Harrison PJ, Warden DR, Johnston C, Refsum H, Smith AD. Polymorphisms in the catechol-O-methyltransferase (COMT) gene influence plasma total homocysteine levels. Am J Med Genet B Neuropsychiatr Genet. 2008 Sep 5;147B(6):996-9.
60. Yakub M, Moti N, Parveen S, Chaudhry B, Azam I, Iqbal MP. Polymorphisms in MTHFR, MS and CBS genes and homocysteine levels in a Pakistani population. PLoS One. 2012;7(3):e33222.
61. Hartley D, Blumenthal T, Carrillo M, DiPaolo G, Esralew L, Gardiner K, et al. Down syndrome and Alzheimer's disease: Common pathways, common goals. Alzheimers Dement. 2014 Dec 12.
62. Ryu YS, Park SY, Jung MS, Yoon SH, Kwen MY, Lee SY, et al. Dyrk1A-mediated phosphorylation of Presenilin 1: a functional link between Down syndrome and Alzheimer's disease. J Neurochem. 2010 Nov;115(3):574-84.
63. Lott IT. Neurological phenotypes for Down syndrome across the life span. Prog Brain Res. 2012;197:101-21.
64. Trazzi S, Fuchs C, De Franceschi M, Mitrugno VM, Bartesaghi R, Ciani E. APP-dependent alteration of GSK3beta activity impairs neurogenesis in the Ts65Dn mouse model of Down syndrome. Neurobiol Dis. 2014 Jul;67:24-36.
65. Trazzi S, Fuchs C, Valli E, Perini G, Bartesaghi R, Ciani E. The triplicated gene APP impairs neuronal precursor differentiation and neurite development through two different domains in the Ts65Dn mouse model for Down syndrome. J Biol Chem. 2013 Jun 5.
66. Trazzi S, Mitrugno VM, Valli E, Fuchs C, Rizzi S, Guidi S, et al. APP-dependent up-regulation of Ptch1 underlies proliferation impairment of neural precursors in Down syndrome. Hum Mol Genet. 2011 Apr 15;20(8):1560-73.
67. Toledo JB, Shaw LM, Trojanowski JQ. Plasma amyloid beta measurements - a desired but elusive Alzheimer's disease biomarker. Alzheimers Res Ther. 2013 Mar 8;5(2):8.
68. Fei M, Jianghua W, Rujuan M, Wei Z, Qian W. The relationship of plasma Abeta levels to dementia in aging individuals

with mild cognitive impairment. J Neurol Sci. 2011 Jun 15;305(1-2):92-6.
69. Blennow K, De Meyer G, Hansson O, Minthon L, Wallin A, Zetterberg H, et al. Evolution of Abeta42 and Abeta40 levels and Abeta42/Abeta40 ratio in plasma during progression of Alzheimer's disease: a multicenter assessment. J Nutr Health Aging. 2009 Mar;13(3):205-8.
70. Lambert JC, Schraen-Maschke S, Richard F, Fievet N, Rouaud O, Berr C, et al. Association of plasma amyloid beta with risk of dementia: the prospective Three-City Study. Neurology. 2009 Sep 15;73(11):847-53.
71. Toledo JB, Vanderstichele H, Figurski M, Aisen PS, Petersen RC, Weiner MW, et al. Factors affecting Abeta plasma levels and their utility as biomarkers in ADNI. Acta Neuropathol. 2011 Oct;122(4):401-13.
72. Chiu MJ, Yang SY, Horng HE, Yang CC, Chen TF, Chieh JJ, et al. Combined plasma biomarkers for diagnosing mild cognition impairment and Alzheimer's disease. ACS Chem Neurosci. 2013 Dec 18;4(12):1530-6.
73. Riedel BC, Thompson PM, Brinton RD. Age, APOE and sex: Triad of risk of Alzheimer's disease. J Steroid Biochem Mol Biol. 2016 Jun;160:134-47.
74. Fukumoto H, Tennis M, Locascio JJ, Hyman BT, Growdon JH, Irizarry MC. Age but not diagnosis is the main predictor of plasma amyloid beta-protein levels. Arch Neurol. 2003 Jul;60(7):958-64.
75. Zimmermann R, Huber E, Schamber C, Lelental N, Mroczko B, Brandner S, et al. Plasma concentrations of the amyloid-beta peptides in young volunteers: the influence of the APOE genotype. J Alzheimers Dis. 2014;40(4):1055-60.
76. Metti AL, Cauley JA, Ayonayon HN, Harris TB, Rosano C, Williamson JD, et al. The demographic and medical correlates of plasma abeta40 and abeta42. Alzheimer Dis Assoc Disord. 2013 Jul-Sep;27(3):244-9.
77. Mehta PD, Capone G, Jewell A, Freedland RL. Increased amyloid beta protein levels in children and adolescents with Down syndrome. J Neurol Sci. 2007 Mar 15;254(1-2):22-7.
78. Mehta PD, Mehta SP, Fedor B, Patrick BA, Emmerling M, Dalton AJ. Plasma amyloid beta protein 1-42 levels are increased in old Down Syndrome but not in young Down Syndrome. Neurosci Lett. 2003 May 22;342(3):155-8.

79. Cavani S, Tamaoka A, Moretti A, Marinelli L, Angelini G, Di Stefano S, et al. Plasma levels of amyloid beta 40 and 42 are independent from ApoE genotype and mental retardation in Down syndrome. Am J Med Genet. 2000 Nov 27;95(3):224-8.
80. Tokuda T, Fukushima T, Ikeda S, Sekijima Y, Shoji S, Yanagisawa N, et al. Plasma levels of amyloid beta proteins Abeta1-40 and Abeta1-42(43) are elevated in Down's syndrome. Ann Neurol. 1997 Feb;41(2):271-3.
81. Mehta PD, Dalton AJ, Mehta SP, Kim KS, Sersen EA, Wisniewski HM. Increased plasma amyloid beta protein 1-42 levels in Down syndrome. Neurosci Lett. 1998 Jan 23;241(1):13-6.
82. Schupf N, Patel B, Silverman W, Zigman WB, Zhong N, Tycko B, et al. Elevated plasma amyloid beta-peptide 1-42 and onset of dementia in adults with Down syndrome. Neurosci Lett. 2001 Apr 6;301(3):199-203.
83. Schupf N, Patel B, Pang D, Zigman WB, Silverman W, Mehta PD, et al. Elevated plasma beta-amyloid peptide Abeta(42) levels, incident dementia, and mortality in Down syndrome. Arch Neurol. 2007 Jul;64(7):1007-13.
84. Coppus AM, Schuur M, Vergeer J, Janssens AC, Oostra BA, Verbeek MM, et al. Plasma beta amyloid and the risk of Alzheimer's disease in Down syndrome. Neurobiol Aging. 2011 Sep 27.
85. Schupf N, Zigman WB, Tang MX, Pang D, Mayeux R, Mehta P, et al. Change in plasma Ass peptides and onset of dementia in adults with Down syndrome. Neurology. 2010 Nov 2;75(18):1639-44.
86. Del Hoyo L, Xicota L, Sánchez G, Cuenca AM, de Sola S, Langohr K, et al. Semantic verbal fluency pattern, dementia rating scores and adaptive behavior correlate with plasma Aβ42 concentrations in Down syndrome young adults. Frontiers in Behavioral Neuroscience. [Original Research]. 2015 2015-November-18;9.
87. Hauser PS, Ryan RO. Impact of apolipoprotein E on Alzheimer's disease. Curr Alzheimer Res. 2013 Oct;10(8):809-17.
88. Mehta PD, Pirttila T, Mehta SP, Sersen EA, Aisen PS, Wisniewski HM. Plasma and cerebrospinal fluid levels of amyloid beta proteins 1-40 and 1-42 in Alzheimer disease. Arch Neurol. 2000 Jan;57(1):100-5.
89. Haddy N, De Bacquer D, Chemaly MM, Maurice M, Ehnholm C, Evans A, et al. The importance of plasma apolipoprotein E concentration in addition to its common polymorphism on inter-

individual variation in lipid levels: results from Apo Europe. Eur J Hum Genet. 2002 Dec;10(12):841-50.
90. Kolovou G, Damaskos D, Anagnostopoulou K, Cokkinos DV. Apolipoprotein E gene polymorphism and gender. Ann Clin Lab Sci. 2009 Spring;39(2):120-33.
91. Phillips MC. Apolipoprotein E isoforms and lipoprotein metabolism. IUBMB Life. 2014 Sep;66(9):616-23.
92. Buonuomo PS, Andrea B, Gerarda M, Annachiara V, Chiara DC, Simona B, et al. Lipid profiles in a large cohort of Italian children with Down Syndrome. Eur J Med Genet. 2016 Jun 22.
93. Adelekan T, Magge S, Shults J, Stallings V, Stettler N. Lipid profiles of children with Down syndrome compared with their siblings. Pediatrics. 2012 Jun;129(6):e1382-7.
94. Licastro F, Dogliotti G, Goi G, Malavazos AE, Chiappelli M, Corsi MM. Oxidated low-density lipoproteins (oxLDL) and peroxides in plasma of Down syndrome patients. Arch Gerontol Geriatr. 2007;44 Suppl 1:225-32.
95. Wang X, Magkos F, Mittendorfer B. Sex differences in lipid and lipoprotein metabolism: it's not just about sex hormones. J Clin Endocrinol Metab. 2011 Apr;96(4):885-93.
96. Chiva-Blanch G, Badimon L, Estruch R. Latest evidence of the effects of the Mediterranean diet in prevention of cardiovascular disease. Curr Atheroscler Rep. 2014 Oct;16(10):446.
97. Estruch R. Anti-inflammatory effects of the Mediterranean diet: the experience of the PREDIMED study. Proc Nutr Soc. 2010 Aug;69(3):333-40.
98. Schreurs BG. The effects of cholesterol on learning and memory. Neurosci Biobehav Rev. 2010 Jul;34(8):1366-79.
99. Corley J, Starr JM, Deary IJ. Serum cholesterol and cognitive functions: the Lothian Birth Cohort 1936. Int Psychogeriatr. 2015 Mar;27(3):439-53.
100. Frisardi V, Panza F, Solfrizzi V, Seripa D, Pilotto A. Plasma lipid disturbances and cognitive decline. J Am Geriatr Soc. 2010 Dec;58(12):2429-30.
101. Panza F, Solfrizzi V, D'Introno A, Colacicco AM, Santamato A, Seripa D, et al. Higher total cholesterol, cognitive decline, and dementia. Neurobiol Aging. 2009 Apr;30(4):546-8.
102. Ancelin ML, Ripoche E, Dupuy AM, Samieri C, Rouaud O, Berr C, et al. Gender-specific associations between lipids and cognitive decline in the elderly. Eur Neuropsychopharmacol. 2014 Jul;24(7):1056-66.

103. Koyama A, Stone K, Yaffe K. Serum oxidized low-density lipoprotein level and risk of cognitive impairment in older women. Neurobiol Aging. 2013 Feb;34(2):634-5 e2.
104. Muldoon MF, Ryan CM, Matthews KA, Manuck SB. Serum cholesterol and intellectual performance. Psychosom Med. 1997 Jul-Aug;59(4):382-7.
105. Taylor AE, Guthrie PA, Smith GD, Golding J, Sattar N, Hingorani AD, et al. IQ, educational attainment, memory and plasma lipids: associations with apolipoprotein E genotype in 5995 children. Biol Psychiatry. 2011 Jul 15;70(2):152-8.
106. Crichton GE, Elias MF, Davey A, Sullivan KJ, Robbins MA. Higher HDL cholesterol is associated with better cognitive function: the Maine-Syracuse study. J Int Neuropsychol Soc. 2014 Nov;20(10):961-70.
107. Campos C, Casado A. Oxidative stress, thyroid dysfunction & Down syndrome. Indian J Med Res. 2015 Aug;142(2):113-9.
108. Iughetti L, Predieri B, Bruzzi P, Predieri F, Vellani G, Madeo SF, et al. Ten-year longitudinal study of thyroid function in children with Down's syndrome. Horm Res Paediatr. 2014;82(2):113-21.
109. Benitez-King G, Riquelme A, Ortiz-Lopez L, Berlanga C, Rodriguez-Verdugo MS, Romo F, et al. A non-invasive method to isolate the neuronal linage from the nasal epithelium from schizophrenic and bipolar diseases. J Neurosci Methods. 2011 Sep 30;201(1):35-45.
110. Duggan CD, Ngai J. Scent of a stem cell. Nat Neurosci. 2007 Jun;10(6):673-4.
111. Murrell W, Feron F, Wetzig A, Cameron N, Splatt K, Bellette B, et al. Multipotent stem cells from adult olfactory mucosa. Dev Dyn. 2005 Jun;233(2):496-515.
112. Borgmann-Winter K, Willard SL, Sinclair D, Mirza N, Turetsky B, Berretta S, et al. Translational potential of olfactory mucosa for the study of neuropsychiatric illness. Transl Psychiatry. 2015;5:e527.
113. Costa AC, Scott-McKean JJ. Prospects for Improving Brain Function in Individuals with Down Syndrome. CNS Drugs. 2013 Jul 4.
114. Gardiner KJ. Molecular basis of pharmacotherapies for cognition in Down syndrome. Trends Pharmacol Sci. 2010 Feb;31(2):66-73.

115. Gardiner KJ. Pharmacological approaches to improving cognitive function in Down syndrome: current status and considerations. Drug Des Devel Ther. 2015;9:103-25.
116. Xicota L, Rodriguez-Morato J, Dierssen M, de la Torre R. Potential Role of (-)-epigallocatechin-3-gallate (EGCG) in the Secondary Prevention of Alzheimer Disease. Curr Drug Targets. 2015 Aug 25.
117. Adayev T, Chen-Hwang MC, Murakami N, Wegiel J, Hwang YW. Kinetic properties of a MNB/DYRK1A mutant suitable for the elucidation of biochemical pathways. Biochemistry. 2006 Oct 3;45(39):12011-9.
118. De la Torre R, De Sola S, Pons M, Duchon A, de Lagran MM, Farre M, et al. Epigallocatechin-3-gallate, a DYRK1A inhibitor, rescues cognitive deficits in Down syndrome mouse models and in humans. Mol Nutr Food Res. 2013 Sep 14.
119. Zheng XX, Xu YL, Li SH, Liu XX, Hui R, Huang XH. Green tea intake lowers fasting serum total and LDL cholesterol in adults: a meta-analysis of 14 randomized controlled trials. Am J Clin Nutr. 2011 Aug;94(2):601-10.
120. Li L, Cao D, Desmond R, Rahman A, Lah JJ, Levey AI, et al. Cognitive performance and plasma levels of homocysteine, vitamin B12, folate and lipids in patients with Alzheimer disease. Dement Geriatr Cogn Disord. 2008;26(4):384-90.
121. Reitz C, Tang MX, Schupf N, Manly JJ, Mayeux R, Luchsinger JA. Association of higher levels of high-density lipoprotein cholesterol in elderly individuals and lower risk of late-onset Alzheimer disease. Arch Neurol. 2010 Dec;67(12):1491-7.
122. Yasuno F, Tanimukai S, Sasaki M, Hidaka S, Ikejima C, Yamashita F, et al. Association between cognitive function and plasma lipids of the elderly after controlling for apolipoprotein E genotype. Am J Geriatr Psychiatry. 2012 Jul;20(7):574-83.
123. Rodriguez-Morato J, Xicota L, Fito M, Farre M, Dierssen M, de la Torre R. Potential role of olive oil phenolic compounds in the prevention of neurodegenerative diseases. Molecules. 2015;20(3):4655-80.
124. Valls-Pedret C, Sala-Vila A, Serra-Mir M, Corella D, de la Torre R, Martinez-Gonzalez MA, et al. Mediterranean Diet and Age-Related Cognitive Decline: A Randomized Clinical Trial. JAMA Intern Med. 2015 Jul;175(7):1094-103.

125. Nishida Y, Akaoka I, Nishizawa T, Maruki M, Maruki K. Hyperlipidaemia in patients with Down's syndrome. Atherosclerosis. 1977 Mar;26(3):369-72.
126. Salo MK, Solakivi-Jaakkola T, Kivimaki T, Nikkari T. Plasma lipids and lipoproteins in Down's syndrome. Scand J Clin Lab Invest. 1979 Sep;39(5):485-90.
127. Zigman WB, Schupf N, Jenkins EC, Urv TK, Tycko B, Silverman W. Cholesterol level, statin use and Alzheimer's disease in adults with Down syndrome. Neurosci Lett. 2007 Apr 18;416(3):279-84.
128. Erba D, Riso P, Bordoni A, Foti P, Biagi PL, Testolin G. Effectiveness of moderate green tea consumption on antioxidative status and plasma lipid profile in humans. J Nutr Biochem. 2005 Mar;16(3):144-9.
129. Maron DJ, Lu GP, Cai NS, Wu ZG, Li YH, Chen H, et al. Cholesterol-lowering effect of a theaflavin-enriched green tea extract: a randomized controlled trial. Arch Intern Med. 2003 Jun 23;163(12):1448-53.
130. Nagao T, Hase T, Tokimitsu I. A green tea extract high in catechins reduces body fat and cardiovascular risks in humans. Obesity (Silver Spring). 2007 Jun;15(6):1473-83.
131. Smith AE, Lockwood CM, Moon JR, Kendall KL, Fukuda DH, Tobkin SE, et al. Physiological effects of caffeine, epigallocatechin-3-gallate, and exercise in overweight and obese women. Appl Physiol Nutr Metab. 2010 Oct;35(5):607-16.
132. Wu AH, Spicer D, Stanczyk FZ, Tseng CC, Yang CS, Pike MC. Effect of 2-month controlled green tea intervention on lipoprotein cholesterol, glucose, and hormone levels in healthy postmenopausal women. Cancer Prev Res (Phila). 2012 Mar;5(3):393-402.
133. Inami S, Takano M, Yamamoto M, Murakami D, Tajika K, Yodogawa K, et al. Tea catechin consumption reduces circulating oxidized low-density lipoprotein. Int Heart J. 2007 Nov;48(6):725-32.
134. Mielgo-Ayuso J, Barrenechea L, Alcorta P, Larrarte E, Margareto J, Labayen I. Effects of dietary supplementation with epigallocatechin-3-gallate on weight loss, energy homeostasis, cardiometabolic risk factors and liver function in obese women: randomised, double-blind, placebo-controlled clinical trial. Br J Nutr. 2014 Apr 14;111(7):1263-71.
135. Miyazaki R, Kotani K, Ayabe M, Tsuzaki K, Shimada J, Sakane N, et al. Minor effects of green tea catechin supplementation on

cardiovascular risk markers in active older people: a randomized controlled trial. Geriatr Gerontol Int. 2013 Jul;13(3):622-9.
136. Nagao T, Meguro S, Hase T, Otsuka K, Komikado M, Tokimitsu I, et al. A catechin-rich beverage improves obesity and blood glucose control in patients with type 2 diabetes. Obesity (Silver Spring). 2009 Feb;17(2):310-7.
137. Princen HM, van Duyvenvoorde W, Buytenhek R, Blonk C, Tijburg LB, Langius JA, et al. No effect of consumption of green and black tea on plasma lipid and antioxidant levels and on LDL oxidation in smokers. Arterioscler Thromb Vasc Biol. 1998 May;18(5):833-41.
138. Ryu OH, Lee J, Lee KW, Kim HY, Seo JA, Kim SG, et al. Effects of green tea consumption on inflammation, insulin resistance and pulse wave velocity in type 2 diabetes patients. Diabetes Res Clin Pract. 2006 Mar;71(3):356-8.
139. van het Hof KH, de Boer HS, Wiseman SA, Lien N, Westrate JA, Tijburg LB. Consumption of green or black tea does not increase resistance of low-density lipoprotein to oxidation in humans. Am J Clin Nutr. 1997 Nov;66(5):1125-32.
140. de Sola S, de la Torre R, Sanchez-Benavides G, Benejam B, Cuenca-Royo A, Del Hoyo L, et al. A new cognitive evaluation battery for Down syndrome and its relevance for clinical trials. Front Psychol. 2015;6:708.
141. Milner B. Interhemispheric differences in the localization of psychological processes in man. Br Med Bull. 1971 Sep;27(3):272-7.
142. Wechsler D. Wechsler Memory Scale (WMS-III). (Third Edit.) ed. San Antonio: TX: The Psychological Corporation; 1997.
143. Benton AL, Hamsher K, Sivan A. Multilingual Aphasia Exam. Iowa City: University of Iowa.1976.
144. Culbertson WC, Zillmer EA. Tower of London Drexel University (TOL DX): Technical manual. (2nd Editio.) ed. New York: Multi-Health Systems Imc.; 2005.
145. Ball SL, Holland AJ, Treppner P, Watson PC, Huppert FA. Executive dysfunction and its association with personality and behaviour changes in the development of Alzheimer's disease in adults with Down syndrome and mild to moderate learning disabilities. Br J Clin Psychol. 2008 Mar;47(Pt 1):1-29.
146. Kaufman AS, Kaufman NL. K-BIT: Kaufman Brief Intelligence Test. Minnesota: American Guidance Service, Ed1990.
147. Harrison P, Oakland T. Adaptive Behavior Assessment System (ABAS-II). (Second Edi.) ed. Los Angeles: PEARSON; 2003.

148. Evenhuis HM, Kengen MMF, Eurlings HAL. Dementia Questionnaire for People with Intellectual Disabilities (DMR). Amsterdam: Harcourt Test Publishers; 2006.
149. Martinez-Gonzalez MA, Garcia-Arellano A, Toledo E, Salas-Salvado J, Buil-Cosiales P, Corella D, et al. A 14-item Mediterranean diet assessment tool and obesity indexes among high-risk subjects: the PREDIMED trial. PLoS One. 2012;7(8):e43134.
150. Schroder H, Fito M, Estruch R, Martinez-Gonzalez MA, Corella D, Salas-Salvado J, et al. A short screener is valid for assessing Mediterranean diet adherence among older Spanish men and women. J Nutr. 2011 Jun;141(6):1140-5.
151. Yousuf A, Khursheed N, Rasool I, Kundal V, Jeelani H, Afroze D. Genetic Variation of ApoE Gene in Ethnic Kashmiri Population and Its Association with Outcome After Traumatic Brain Injury. J Mol Neurosci. 2015 Apr 10.
152. Marioni RE, Campbell A, Scotland G, Hayward C, Porteous DJ, Deary IJ. Differential effects of the APOE e4 allele on different domains of cognitive ability across the life-course. Eur J Hum Genet. 2016 Jun;24(6):919-23.
153. Scarmeas N, Stern Y, Tang MX, Mayeux R, Luchsinger JA. Mediterranean diet and risk for Alzheimer's disease. Ann Neurol. 2006 Jun;59(6):912-21.
154. Scarmeas N, Stern Y, Mayeux R, Manly JJ, Schupf N, Luchsinger JA. Mediterranean diet and mild cognitive impairment. Arch Neurol. 2009 Feb;66(2):216-25.
155. Feart C, Samieri C, Rondeau V, Amieva H, Portet F, Dartigues JF, et al. Adherence to a Mediterranean diet, cognitive decline, and risk of dementia. JAMA. 2009 Aug 12;302(6):638-48.
156. Martinez-Lapiscina EH, Clavero P, Toledo E, San Julian B, Sanchez-Tainta A, Corella D, et al. Virgin olive oil supplementation and long-term cognition: the PREDIMED-NAVARRA randomized, trial. J Nutr Health Aging. 2013;17(6):544-52.
157. Droge W, Schipper HM. Oxidative stress and aberrant signaling in aging and cognitive decline. Aging Cell. 2007 Jun;6(3):361-70.
158. Leiva E, Mujica V, Sepulveda P, Guzman L, Nunez S, Orrego R, et al. High levels of iron status and oxidative stress in patients with metabolic syndrome. Biol Trace Elem Res. 2013 Jan;151(1):1-8.
159. Obeid R, Hartmuth K, Herrmann W, Gortner L, Rohrer TR, Geisel J, et al. Blood biomarkers of methylation in Down syndrome

and metabolic simulations using a mathematical model. Mol Nutr Food Res. 2012 Oct;56(10):1582-9.
160. Gueant JL, Anello G, Bosco P, Gueant-Rodriguez RM, Romano A, Barone C, et al. Homocysteine and related genetic polymorphisms in Down's syndrome IQ. J Neurol Neurosurg Psychiatry. 2005 May;76(5):706-9.
161. Fukuda N, Hamajima N, Wakai K, Suzuki K. A cross-sectional study to find out the relationship of methylenetetrahydrofolate reductase (MTHFR) C677T genotype with plasma levels of folate and total homocysteine by daily folate intake in Japanese. J Nutr Sci Vitaminol (Tokyo). 2014;60(4):231-8.
162. Semmler A, Moskau-Hartmann S, Stoffel-Wagner B, Elger C, Linnebank M. Homocysteine plasma levels in patients treated with antiepileptic drugs depend on folate and vitamin B12 serum levels, but not on genetic variants of homocysteine metabolism. Clin Chem Lab Med. 2013 Mar 1;51(3):665-9.
163. Biselli JM, Goloni-Bertollo EM, Haddad R, Eberlin MN, Pavarino-Bertelli EC. The MTR A2756G polymorphism is associated with an increase of plasma homocysteine concentration in Brazilian individuals with Down syndrome. Braz J Med Biol Res. 2008 Jan;41(1):34-40.
164. De Lorenzo A, Noce A, Bigioni M, Calabrese V, Della Rocca DG, Di Daniele N, et al. The effects of Italian Mediterranean organic diet (IMOD) on health status. Curr Pharm Des. 2010;16(7):814-24.
165. Carter SJ, Roberts MB, Salter J, Eaton CB. Relationship between Mediterranean Diet Score and atherothrombotic risk: findings from the Third National Health and Nutrition Examination Survey (NHANES III), 1988-1994. Atherosclerosis. 2010 Jun;210(2):630-6.
166. Verdejo-Garcia A, Fagundo AB, Cuenca A, Rodriguez J, Cuyas E, Langohr K, et al. COMT val158met and 5-HTTLPR genetic polymorphisms moderate executive control in cannabis users. Neuropsychopharmacology. 2013 Jul;38(8):1598-606.
167. Panagiotakos DB, Pitsavos C, Zeimbekis A, Chrysohoou C, Stefanadis C. The association between lifestyle-related factors and plasma homocysteine levels in healthy individuals from the "ATTICA" Study. Int J Cardiol. 2005 Feb 28;98(3):471-7.
168. Ayesa-Arriola R, Perez-Iglesias R, Rodriguez-Sanchez JM, Mata I, Gomez-Ruiz E, Garcia-Unzueta M, et al. Homocysteine and cognition in first-episode psychosis patients. Eur Arch Psychiatry Clin Neurosci. 2012 Oct;262(7):557-64.

169. Choe YM, Sohn BK, Choi HJ, Byun MS, Seo EH, Han JY, et al. Association of homocysteine with hippocampal volume independent of cerebral amyloid and vascular burden. Neurobiol Aging. 2014 Jul;35(7):1519-25.
170. Alexopoulos P, Lehrl S, Richter-Schmidinger T, Kreusslein A, Hauenstein T, Bayerl F, et al. Short-term influence of elevation of plasma homocysteine levels on cognitive function in young healthy adults. J Nutr Health Aging. 2010 Apr;14(4):283-7.
171. de Jager CA. Critical levels of brain atrophy associated with homocysteine and cognitive decline. Neurobiol Aging. 2014 May 15.
172. Rumble B, Retallack R, Hilbich C, Simms G, Multhaup G, Martins R, et al. Amyloid A4 Protein and Its Precursor in Down's Syndrome and Alzheimer's Disease. New England Journal of Medicine. 1989;320(22):1446-52.
173. Lott IT, Head E. Down syndrome and Alzheimer's disease: a link between development and aging. Ment Retard Dev Disabil Res Rev. 2001;7(3):172-8.
174. Meng X, D'Arcy C. Education and dementia in the context of the cognitive reserve hypothesis: a systematic review with meta-analyses and qualitative analyses. PLoS One. 2012;7(6):e38268.
175. Seifan A, Schelke M, Obeng-Aduasare Y, Isaacson R. Early Life Epidemiology of Alzheimer's Disease - A Critical Review. Neuroepidemiology. 2015 Dec;45(4):237-54.
176. Stern Y. Cognitive reserve in ageing and Alzheimer's disease. Lancet Neurol. 2012 Nov;11(11):1006-12.
177. Yasuno F, Kazui H, Morita N, Kajimoto K, Ihara M, Taguchi A, et al. Low amyloid-beta deposition correlates with high education in cognitively normal older adults: a pilot study. Int J Geriatr Psychiatry. 2014 Nov 26.
178. Wirth M, Villeneuve S, La Joie R, Marks SM, Jagust WJ. Gene-environment interactions: lifetime cognitive activity, APOE genotype, and beta-amyloid burden. J Neurosci. 2014 Jun 18;34(25):8612-7.
179. Almeida RP, Schultz SA, Austin BP, Boots EA, Dowling NM, Gleason CE, et al. Effect of Cognitive Reserve on Age-Related Changes in Cerebrospinal Fluid Biomarkers of Alzheimer Disease. JAMA Neurol. 2015 Jun;72(6):699-706.
180. Yaffe K, Weston A, Graff-Radford NR, Satterfield S, Simonsick EM, Younkin SG, et al. Association of plasma beta-amyloid level and cognitive reserve with subsequent cognitive decline. JAMA. 2011 Jan 19;305(3):261-6.

181. Okereke OI, Selkoe DJ, Grodstein F. Plasma beta-amyloid level, cognitive reserve, and cognitive decline. JAMA. 2011 Apr 27;305(16):1655; author reply -6.
182. Okereke OI, Xia W, Selkoe DJ, Grodstein F. Ten-year change in plasma amyloid beta levels and late-life cognitive decline. Arch Neurol. 2009 Oct;66(10):1247-53.
183. Temple V, Jozsvai E, Konstantareas MM, Hewitt TA. Alzheimer dementia in Down's syndrome: the relevance of cognitive ability. J Intellect Disabil Res. 2001 Feb;45(Pt 1):47-55.
184. Jack CR, Jr., Knopman DS, Jagust WJ, Petersen RC, Weiner MW, Aisen PS, et al. Tracking pathophysiological processes in Alzheimer's disease: an updated hypothetical model of dynamic biomarkers. Lancet Neurol. 2013 Feb;12(2):207-16.
185. Fagundo AB, Cuyas E, Verdejo-Garcia A, Khymenets O, Langohr K, Martin-Santos R, et al. The influence of 5-HTT and COMT genotypes on verbal fluency in ecstasy users. J Psychopharmacol. 2010 Sep;24(9):1381-93.
186. Head E, Doran E, Nistor M, Hill M, Schmitt FA, Haier RJ, et al. Plasma amyloid-beta as a function of age, level of intellectual disability, and presence of dementia in Down syndrome. J Alzheimers Dis. 2011;23(3):399-409.
187. Prasher VP, Sajith SG, Mehta P, Zigman WB, Schupf N. Plasma beta-amyloid and duration of Alzheimer's disease in adults with Down syndrome. Int J Geriatr Psychiatry. 2010 Feb;25(2):202-7.
188. Lai F, Williams RS. A prospective study of Alzheimer disease in Down syndrome. Arch Neurol. 1989 Aug;46(8):849-53.
189. Deb S, Braganza J. Comparison of rating scales for the diagnosis of dementia in adults with Down's syndrome. J Intellect Disabil Res. 1999 Oct;43 (Pt 5):400-7.
190. Farrer MJ, Crayton L, Davies GE, Oliver C, Powell J, Holland AJ, et al. Allelic variability in D21S11, but not in APP or APOE, is associated with cognitive decline in Down syndrome. Neuroreport. 1997 May 6;8(7):1645-9.
191. Del Bo R, Comi GP, Bresolin N, Castelli E, Conti E, Degiuli A, et al. The apolipoprotein E epsilon4 allele causes a faster decline of cognitive performances in Down's syndrome subjects. J Neurol Sci. 1997 Jan;145(1):87-91.
192. Patel A, Rees SD, Kelly MA, Bain SC, Barnett AH, Thalitaya D, et al. Association of variants within APOE, SORL1, RUNX1, BACE1 and ALDH18A1 with dementia in Alzheimer's disease in

subjects with Down syndrome. Neurosci Lett. 2011 Jan 7;487(2):144-8.
193. Medvedev SP, Shevchenko AI, Zakian SM. Induced Pluripotent Stem Cells: Problems and Advantages when Applying them in Regenerative Medicine. Acta naturae. 2010 Jul;2(2):18-28.
194. Tajinda K, Ishizuka K, Colantuoni C, Morita M, Winicki J, Le C, et al. Neuronal biomarkers from patients with mental illnesses: a novel method through nasal biopsy combined with laser-captured microdissection. Mol Psychiatry. 2010 Mar;15(3):231-2.
195. Benitez-King G, Valdes-Tovar M, Trueta C, Galvan-Arrieta T, Argueta J, Alarcon S, et al. The microtubular cytoskeleton of olfactory neurons derived from patients with schizophrenia or with bipolar disorder: Implications for biomarker characterization, neuronal physiology and pharmacological screening. Molecular and cellular neurosciences. 2016 Jun;73:84-95.
196. Palacios-Reyes CP, Espinosa AE, Contreras AC, Ordonez RO, Hidalgo-Miranda AH, Rubio-Gayosso IR, et al. Williams neural stem cells: new model for insight into microRNA dysregulation. Front Biosci (Elite Ed). 2013;E5:1057-73.

Annex 1

de la Torre R, de Sola S, Hernandez G, Farré M, Pujol J, Rodriguez J, et al. Safety and efficacy of cognitive training plus epigallocatechin-3-gallate in young adults with Down's syndrome (TESDAD): a double-blind, randomised, placebo-controlled, phase 2 trial. The Lancet Neurology. 2016;15(8):801-10. . DOI: 10.1016/S1474-4422(16)30034-5

Annex 2

Xicota L, Rodriguez-Morato J, Dierssen M, de la Torre R. Potential Role of (-)-epigallocatechin-3-gallate (EGCG) in the Secondary Prevention of Alzheimer Disease. Curr Drug Targets. 2017 Dec 29;18(2):174–95. DOI: 10.2174/1389450116666150825113655

Annex 3: Mediterranean Diet Compliance Questionnaire (PREDIMED)

CUESTIONARIO DE ADHESIÓN A LA DIETA MEDITERRÁNEA (14 PUNTOS DE PREDIMED, SÓLO GRUPO CONTROL)

1. ¿Usa usted el aceite de oliva como principal grasa para cocinar?:	Si= 1 punto	☐
2. ¿Cuánto aceite de oliva consume en total al día?: (incluyendo el usado para freír, comidas fuera de casa, ensaladas, etc.)	4 o más cucharadas= 1 punto	☐
3. ¿Cuántas raciones de verdura u hortalizas consume al día?: (las guarniciones o acompañamientos= 1/2 ración)1 ración= 200 g.	2 o más (al menos una de ellas en ensalada o crudas)= 1 punto	☐
4. ¿Cuántas piezas de fruta (incluyendo zumo natural) consume al día?:	3 o más al día= 1 punto	☐
5. ¿Cuántas raciones de carnes rojas, hamburguesas, salchichas o embutidos consume al día?: (ración= 100 - 150 g.)	Menos de 1 al día= 1 punto	☐
6. ¿Cuántas raciones de mantequilla, margarina o nata consume al día?: (porción individual=12 g.)	Menos de 1 al día= 1 punto	☐
7. ¿Cuántas bebidas carbonatadas y/o azucaradas (refrescos, colas, tónicas, bitter) consume al dia?:	Menos de 1 al día= 1 punto	☐
8. ¿Bebe usted vino? ¿Cuánto consume a la semana?:	7 o más vasos a la semana= 1 punto	☐
9. ¿Cuántas raciones de legumbres consume a la semana?: (1 plato o ración de 150 g.)	3 o más a la semana= 1 punto	☐
10. ¿Cuántas raciones de pescado - mariscos consume a la semana?: (1 plato, pieza o ración= 100 - 150 g. de pescado o 4 - 5 piezas o 200 g. de marisco)	3 o más a la semana= 1 punto	☐
11. ¿Cuántas veces consume repostería comercial (no casera) como galletas, flanes, dulces o pasteles a la semana?:	Menos de 2 a la semana= 1 punto	☐
12. ¿Cuántas veces consume frutos secos a la semana?: (ración 30 g.)	3 o más a la semana= 1 punto	☐
13. ¿Consume usted preferentemente carne de pollo, pavo o conejo en vez de ternera, cerdo, hamburguesas o salchichas?: (carne de pollo= 1 pieza o ración de 100 - 150 g.)	Si= 1 punto	☐
14. ¿Cuántas veces a la semana consume los vegetales cocinados, la pasta, arroz u otros platos aderezados con salsa de tomate, ajo, cebolla o puerro elaborada a fuego lento con aceite de oliva (sofrito)?:	2 o más a la semana= 1 punto	☐
PUNTUACIÓN TOTAL:		☐0

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Nenugalėk. *Do not defeat.*
Nesigink. *Do not fight back.*
Nepasiduok. *Do not surrender.*

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Aquesta tesi s'ha imprès gràcies al ajut econòmic de la Fundació IMIM.