Universidad de Navarra

Facultad de Farmacia y Nutrición

“Deciphering the connection between aging, insulin resistance and cognitive decline in Alzheimer’s disease: role of TMAO as linking mechanism”

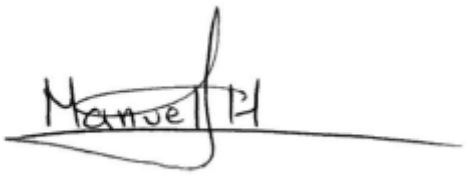
Manuel Humberto Janeiro Arenas

Pamplona, 2021

Universidad de Navarra

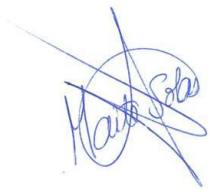
Facultad de Farmacia y Nutrición

Memoria presentada por Don Manuel Humberto Janeiro Arenas para aspirar al grado de Doctor por la Universidad de Navarra.



Fdo. Manuel Humberto Janeiro Arenas

El presente trabajo ha sido realizado bajo nuestra dirección en el **Departamento de Farmacología y Toxicología** de la Facultad de Farmacia y Nutrición de la Universidad de Navarra y autorizamos su presentación ante el Tribunal que lo ha de juzgar.



VºBº Directores

Dra. María Javier Ramírez Gil

Dra. Maite Solas Zubiaurre

Este trabajo ha sido posible gracias a la financiación del Ministerio de Universidades y la Universidad de Navarra. La investigación que ha dado lugar a estos resultados ha sido impulsada por la ayuda predoctoral FPU 2016-2021.

A mis abuelos

“La paciencia y la diligencia, como la fe, mueven montañas”

William Penn

AGRADECIMIENTOS

Con estas palabras me gustaría agradecer a todas las personas que de una manera u otra han contribuido al desarrollo de esta tesis y han hecho posible la realización de la misma. No ha sido un camino fácil, tanto por los obstáculos que lleva implícita la misma ciencia, como por las circunstancias externas que han ido surgiendo; pero sí es un camino necesario para alcanzar mi meta de ser docente. Sin duda, el esfuerzo ha merecido la pena y haber recorrido este camino con ustedes ha hecho que este camino sea inolvidable y lleno de experiencias de valor incalculable para lo que está por venir.

Me gustaría comenzar dando las gracias a la Universidad de Navarra por haberme formado como Farmacéutico y Nutricionista, pero también como persona.

La Universidad de Navarra es quién me ha formado como profesional, pero si realmente tuviera que agradecer a alguien el estar hoy aquí sería a mis abuelos. Mis abuelos han sido una parte muy importante de mi vida y sin su ayuda, no habría podido venir a esta Universidad. Quiero agradecer a mis abuelos Joaquina y Pepe, que ya no están pero que siempre los tengo muy presentes. Mi abuelo Pepe me enseñó a aspirar alto y a trabajar duro para conseguir lo que te propones y mi abuela Joaquina que por muy mal que se tornen las cosas siempre se puede sonreír a la vida. De ella he heredado, su simpatía, su alegría ante la vida y sus "fullerías" en los juegos de mesa, cosa que también ha heredado mi madre. Por cosas del destino, después de varios infartos cerebrales, pasó sus últimos años con demencia e hizo que empezara a interesarme por la neurociencia. No quería volver a pasar por esa situación de nuevo, aunque inevitablemente he tenido que volver a pasar por ello y probablemente me vuelva a tocar pasar por una situación similar. Quería extender mi agradecimiento a mis otras dos mitades, mis abuelos Manolo y María, que me han ayudado a no tirar la toalla nunca ni a perder la esperanza. Mi abuelo Manolo siempre ha confiado en mí y en mi potencial. Nunca ha dudado en hacer todo lo posible para que llegue lo más lejos posible, siempre se ha sacrificado por sacar la familia adelante y porque continúe formándome y ampliando mi conocimiento. Estos últimos años la demencia derivada de su enfermedad de Parkinson había comenzado a aparecer, pero ello no borraba su sonrisa de la cara, la preocupación por mis estudios, ni sus ganas de trabajar para sacar a su familia adelante, queriendo ir a entregar las cartas del Correo a los 88 años, no vaya a ser que lo despidieran... Lamentablemente, hemos tenido que decirle adiós hace apenas unos meses pero siempre voy a recordarle y a agradecerle todo lo que me ha enseñado. Por último, mi abuela María es la que me ha aportado el don de la prudencia y de la paciencia, dos bienes muy preciados para la vida, pero también para la ciencia, porque como bien dicen "la paciencia es la reina de la ciencia" y no hay que desistir aunque las cosas se tuerzan.

Quisiera continuar dando las gracias a mi directora de tesis, Mariaja, por haberme permitido hacer el TFG con ella y luego animarme a realizar esta tesis en el Departamento de Farmacología y Toxicología. Gracias por tu serenidad ante las adversidades y tu confianza en que todo tiene solución; por guiarme, compartir tus

conocimientos y dirigir esta tesis. La verdad es que quedarme en Pamplona aunque me llamen más los lugares más cálidos, fue una decisión bastante fácil porque sabía que imperaba el buen ambiente en este departamento y ya conocía a la mayoría tras haber realizado mi TFG aquí. Indudablemente nuestro "Depar" está formado por personas maravillosas que hacen todo más fácil.

Gracias Mariaja y Maite por vuestra comprensión y preocupación ante las diferentes dificultades que me han ido surgiendo a lo largo de la tesis. No han sido momentos fáciles, pero han sido más llevaderos con vuestra ayuda. Gracias Maite por codirigir esta tesis, por aguantar mis chapas de estadística, por ayudarme y guiarme cuando ya no sabía por dónde ir y por inculcarme el interés de tener la certeza de que lo que está hecho efectivamente esté bien hecho. Gracias a ambas por vuestra cercanía, vuestro cariño y la confianza que habéis depositado en mí.

Continuando con el Depar, me gustaría darte las gracias Rosa y destacar tu alegría y tu ingenuidad, por un motivo u otro siempre conseguías arrancarme una sonrisa de la cara. Gracias por contar siempre conmigo para organizar eventos, asistir a charlas y congresos o incluso preparar una pequeña clase. Gracias Pepe por hacerme salir a correr, por tu saber estar y por compartir con nosotros tus reflexiones. Extender mi agradecimiento a Bea y Guada, que me han permitido explayarme y mostrar mis dotes artísticas en las prácticas de EIP y Atención Farmacéutica. Gracias por vuestra empatía y comprensión, por estar siempre dispuestas a ayudar y gracias por resolver mis dudas científicas y mundanas y por preocuparos por mí. Gracias Bea por hacer de madre y a la vez hacer de doctoranda encubierta. A Elena gracias por a pesar de su ritmo de vida frenético, pararse siempre a preguntar qué tal nos va la vida y ser la primera dispuesta a echar una mano aunque no tenga tiempo ni para comer. Gracias también por tus pésimas imitaciones sobre mí, que siempre me hacían reír pero que aún tienes que pulir mucho, sobre todo el acento. A Mikel, por ayudarme siempre, no sin antes quejarse o llamarme post-millenial a pesar de que seamos de la misma generación… Gracias por las quedadas con churros, por los acertijos y juegos y por resolverme tantas dudas y enseñarme tanto, tanto del laboratorio como de la vida.

Apartado especial para Sandra, que ha sido muy importante para mí durante el desarrollo de esta tesis. Persona encantadora y polifacética, lo mismo te ayuda a terminar un experimento que te lee el futuro con su péndulo. Gracias por tantas risas y momentos divertidos, gracias por ser como una amiga y por cuidarme como un hijo. Gracias por animarme en mis momentos de bajón, por escuchar mis problemas y tratar de buscarles solución o restarle hierro al asunto y especialmente gracias por compartir tus conocimientos científicos y no tan científicos conmigo, porque al fin y al cabo los cafés son para desconectar y hay que saber de todo, que si no, es imposible acertar el quesito rosa del trivial y ganar la partida.

No podría dejar de agradecer los buenos consejos y momentos de los que han compartido parte del camino conmigo y que ahora ya son doctores y han emprendido nuevos caminos. Gracias Silvia e Hilda por ponerme al día de todo lo que se cuece en el

Depar y explicarme las nociones básicas de lo que iba a ser este camino, que tiene sus dificultades pero también sus recompensas. Gracias a Borja y Teresa por las risas y los momentos de desconexión, ahora soy yo el que tiene que acabar este camino y emprender uno nuevo.

Este es el ciclo de las tesis, cuando ya te has acomodado es hora de partir hacia nuevos retos, aunque siempre quedarán los recuerdos. El Depar se queda en buenas manos, gracias Carlos por tus conocimientos y mostrarte siempre dispuesto a echar una mano, creo que eres el ejemplo de que las apariencias engañan porque el que tiene la suerte de conocerte sabe que eres una persona encantadora y sin malicia alguna. Gracias Noemí por ser como tú eres, aunque te identifiques más con un erizo, eres una persona muy achuchable y risueña. Sólo hay que evitar que estés hambrienta. Además, eres muy resolutiva y con muy buenas dotes "investigadoras" para todo. Gracias Jinya por tu simpatía y por tu amabilidad, por tus historias y por tus regalos. Gracias también a mi compañera de bromas y locuras, Ainhoa, que ha revolucionado el departamento pero de buena manera y es que en un mundo de locos tener sentido no tiene sentido. Apartado especial merece mi Mary, que he compartido con ella toda mi aventura desde que llegué a Pamplona, es un alma pura, maravillosa persona y estoy muy feliz de haberte conocido y que seamos amigos, eres mi alma complementaria, tan parecidos y a la vez tan diferentes. Hemos compartido mucho y me has ayudado mucho en todos los sentidos de la vida y estoy seguro de que seguiremos compartiendo muchas cosas más. Por último, gracias también a Amaia que es la recién llegada, pero estoy seguro de que cuidará muy bien de todos.

Gracias también a Amaia Azqueta por ayudarme a entender los ensayos de diferenciación de adipocitos, a Josune Orbe por dejarnos un vial con células de barrera hematoencefálica, a Itziar y Antonio Pineda por ayudarnos con las mediciones de TMAO y a Fermín y José Ignacio por ayudarnos con la recolección de las muestras de microbiota y enseñarme a manejar el programa estadístico para su correcto estudio.

Thank you Dr. Ping Yip and Professor Adina Michael-Titus for letting me stay at Blizard Institute from Queen Mary University of London and learn so many new techniques. Thanks Ping for your availability and for sharing your knowledge with me and thanks both again for the facilities you gave me in spite of the difficulties originated from the Administration and the COVID-19 situation. Gracias también a Adri, Elena y Laura por acogerme durante mi estancia en Londres y compartir tan buenos momentos juntos.

Quiero también agradecer el apoyo de mis amigos de "Los Barrios City" (Ballest, Kevin, Gallardo, Pachi, Ana Salazar y Jairo). Es increíble que a pesar de la distancia cuando nos encontramos en vacaciones sea como si nunca nos hubiéramos separado. Pero quiero dar gracias en especial a Sara, Ana Piñer, Emilio, Mayara y Moya, por ser los que más se han preocupado por mi tesis y por mí, pero principalmente por estar ahí para escuchar mis dramas y ser un hombro virtual donde apoyarse. Gracias Emilio por poner de tu parte y hacer el esfuerzo por ser más empático y comprensivo y estar ahí a

tu manera, si antes decías que tu asignatura pendiente eran las relaciones personales ahora te doy el aprobado y casi rozas el notable XD. Gracias Sara y Ana por sacar tiempo para hablar, contarnos "noticias frescas" y pasar tiempo juntos a pesar de estar siempre superocupadas, sé que siempre puedo contar con ustedes ante cualquier problema.

Gracias también a los miembros del GMU porque hemos conseguido seguir en contacto y estar al día de todas las novedades científicas y no tan científicas aunque hayamos acabado la carrera. Gracias Patri, Amaia, Amalli, Isa, Lidia, Lucía, Bea, Vivian, Espe, Jacobo y de nuevo a María por haber compartido tantos buenos momentos durante la carrera y después de ella, por esas escapadas a Acedo y León y en general por estos 10 años que hace ya que nos conocemos, que se dice rápido. Gracias por estar ahí en los momentos difíciles y por resolver tan rápidamente cualquier duda farmacéutica que surge. Gracias Jacobo por estar siempre ahí estos 10 años y por presentarme a tu cuadrilla y especialmente por sacar un ratito para estar conmigo en mi cumpleaños a pesar de la situación en la que te encontrabas. Es un gesto que aprecio mucho viniendo de ti y de todo lo que te rodeaba.

Gracias también a mis amigos del Cheddar, por acogerme cuando se fueron todos mis amigos de la carrera, por hacerme más fácil vivir en Pamplona estos años de tesis y hacerme sentir uno más de la cuadrilla para no echar tanto de menos Andalucía. Mención especial para Asier por ser tan generoso, sorprenderme con una entrada para ver al Betis y por ser a menudo mi compañero de fedeo, a Fedez por aceptar siempre una invitación a dar una vuelta y para Borja por sorprenderme cada finde en este último período de la tesis con una escapada al monte para desconectar de experimentos y dramas; quién nos iba a decir que íbamos a congeniar tanto después de ese desastroso día en que nos conocimos…

No puedo olvidarme de extender mi agradecimiento a mis amigos del Discord que han sido mi segunda familia durante el confinamiento, cuando me quedé atrapado en Pamplona y no tenía nadie con quien hablar físicamente. Gracias Jacobo, Asier, Álvaro, Miguel, Joanes, Unai, Jungo, Carmen e incluso a Gil. Gracias por vuestros consejos y por tantas y tan diversas conversaciones que hemos tenido. Habéis sido mi apoyo durante el confinamiento y mi vía de escape después del trabajo. Sin vosotros no hubiera sido lo mismo. Gracias también a Guzmán por acogerme en su piso durante esta última etapa de la tesis, interesarse por mi trabajo y compartir estampadas en AmT.

Natalia se merece un apartado especial en estos agradecimientos. Conocerla fue todo un soplo de aire fresco en mi vida, la pandemia no trajo sólo cosas malas. Aunque hace poco más de un año que nos conocimos, siento como si siempre hubiera estado ahí, formando parte de mi vida y es que es la persona más parecida a mí que he conocido y me alegra saber que puedo contar con ella. Gracias también por presentarme a Loli y a Marcos, dos personas encantadoras con las que comparto la pasión por los juegos de mesa y con los que el sólo hecho de escuchar sus nombres viene acompañado de risas aseguradas.

Gracias a mi tía Mamen por acogerme en su casa y ayudarme con el alquiler. Gracias por preocuparte casi diariamente por mí, rozando casi el punto de la pesadez en algunos momentos, pero te comprendo porque somos muy parecidos. Gracias por haberme ayudado de tantas maneras diferentes y por hacerme sentir tu sobrino preferido (aunque no tengas más jajaja). Gracias también a mi tía Lalo por interesarse por mí y mandar esos chistes tan malos por el chat de familia, que te ríes pero de lo malo que son.

Gracias a mi tía Inma por invitarme a comer los fines de semana y por ayudarme con las maletas y las cosas de la casa siempre que lo he necesitado. Tener a alguien de la familia cerca siempre es bueno para que la distancia que me separa de mis padres no se haga tan difícil. Gracias a mis primas María y Paula por hacerme la estancia más cómoda aquí, pero en especial a Rocío, mi compañera de cante, baile y juegos; y mi alumna predilecta. Ha sido un placer ayudarte a estudiar y repasar mis conocimientos de estadística contigo y al final ha merecido la pena porque lo has bordado en estadística. Además, eres una persona superagradecida por lo que no me arrepiento nada de invertir parte de mi tiempo libre estudiando contigo, pues al fin y al cabo estaba haciendo una de las cosas que más me gusta que es ser docente y a la vez mantenía frescos mis conocimientos de estadística.

Por último, quería terminar dando las gracias a mi familia más directa, mis padres y mi hermana. Sin ellos no estaría aquí, son los que me han criado junto con mis abuelos y sin su ayuda ni sus valores no sería la persona que soy hoy, ni estaría donde estoy. Ha sido muy difícil estar tanto tiempo en la lejanía y no poder ayudaros más en los problemas por los que habéis pasado. Quería agradecer a mi padre su ayuda para escucharme siempre que ha estado disponible, las charlas contigo siempre me ayudaban a afrontar el día y a seguir adelante, aunque me hubiera gustado que fueran más frecuentes, pero sé que no ha podido ser por diversas circunstancias. Gracias mamá por tu entereza y tu paciencia. Eres la persona más fuerte que conozco y el vivo ejemplo de que se puede con todo, por muy grande que sean los obstáculos que estén por venir, si nos mantenemos unidos aunque sea en la distancia, se podrán superar. Gracias por intentar protegerme y hacer lo imposible para que no me enterara de lo que sucedía por allí, sé que lo hacías para que pudiera sacar adelante esta tesis, pero ya sabes que prefiero estar al tanto de todo, sea bueno o malo. Finalmente, quería darle las gracias a mi hermana. Gracias por pensar en mí cuando creías que nada tenía solución. Gracias por ser mi hermana y compartir tan buenos momentos conmigo y a pesar de que nos peleemos como hacen todos los hermanos, volver luego a estar de risas como siempre. Gracias por último por entender que a pesar de tus ideales, la experimentación es necesaria para el desarrollo de nuevos medicamentos y poder tener una mejor calidad de vida. Los amigos van y vienen pero la familia es lo que perdura y permanece inalterable a través de los tiempos.

De nuevo, gracias a todos y cada uno de ustedes por haber contribuido a la realización de esta tesis, que es el primer paso para el desarrollo de mi carrera investigadora y docente.

ABBREVIATIONS

Aβ: Amyloid-β

AD: Alzheimer's Disease

APP: β-Amyloid Precursor Protein

AUC: Area Under Curve

BBB: Blood Brain Barrier

βCTF: β-cleaved Carboxy-Terminal Fragment of β-Amyloid Precursor Protein

CBS: Calf Bovine Serum

CKD: Chronic Kidney Disease

CNS: Central Nervous System

CVD: Cardiovascular Disease

CSF: Cerebrospinal Fluid

DMA: Dimethylamine

DMB: 3,3-dimethyl-1-butanol

eNOS: endothelial Nitric Oxide Synthase

ER: Endoplasmic Reticulum

FBS: Fetal Bovine Serum

FOS: Fructooligosaccharide

FMO1: Flavin Monooxygenase I

FMO3: Flavin Monooxygenase III

GALT: Gut-Associated Lymphoid Tissues

GBB: γ-Butyrobetaine

GF: Germ Free

GFAP: Glial Fibrillary Acidic Protein

GFR: Glomerular Filtration Rate

GI: Gastrointestinal

GTT: Glucose Tolerance Test

HC: Healthy Control

HCHO: Formaldehyde

HFD: High Fat Diet

HFHS: High Fat High Sugar

HOMA: Homeostatic Model Assessment

IDP: Intrinsically Disordered Proteins

ITT: Insulin Tolerance Test

JNK: c Jun-N-terminal Kinase

LPS: Lipopolysaccharide

MCI: Mild Cognitive Impairment

MJD/SCA-3: Machado-Joseph Disease/Spinocerebellar Ataxia-3

MWM: Morris Water Maze

NLRP3: NLR family Pyrin domain containing 3

NO: Nitric Oxide

NORT: Novel Object Recognition Test

OD: Optical Density

p-Tau: phosphorylated Tau

PD: Parkinson's disease

PFA: Paraformaldehyde

PrPC: Scrapie Prion Protein

PrPSc: Pathogenic isoform of Scrapie Prion Protein

PS1: Presenilin 1

SAMP8: Senescence Accelerated Mouse-Prone 8

SAMR1: Senescence Accelerated Mouse-Resistant 1

t-Tau: total Tau

T2DM: Type 2 Diabetes Mellitus

TLR4: Toll-like receptor 4

TMA: Trimethylamine

TMADH: Trimethylamine dehydrogenase

TMAO: Trimethylamine N-oxide

TNF-α: Tumor Necrosis Factor Alpha

WAT: White adipose Tissue

WB: Western Blot

ZO-1: Zonulin

TABLE OF CONTENTS

ABSTRACT

ABSTRACT

Insulin resistance and neurodegenerative diseases such as Alzheimer's disease are considered part of the main threats to health in old age. In light of the high numbers of overweight/obese and diabetic individuals, there is a clear need to better understand the pathophysiological mechanisms underpinning insulin resistance/obesity and the accompanying impact on cognitive function. The aim of the present study was to investigate the missing link between aging, insulin resistance and cognitive decline as aging and insulin resistance are both risk factors for Alzheimer's disease.

TMA is a gut metabolite which proceeds from the bacterial synthesis of substrates such as L-carnitine and choline. TMA is then rapidly further oxidized by hepatic flavin monooxygenases FMO3 and FMO1 to form TMAO. At first, TMAO was thought to be a waste product of choline metabolism without action in our organism, but nowadays, there is emerging evidence linking TMAO to atherosclerosis, systemic inflammation, type 2 diabetes mellitus and even neuropathologies.

Plasma TMAO levels show wide inter- and intra-individual variations. These levels are influenced by several factors but the main factor influencing TMAO levels is aging. Some studies performed in human and rats have revealed that plasma TMAO levels are closely related to aging showing increasing levels with age.

In this context, we have investigated if TMAO could be the link between metabolic diseases and cognitive deficiencies. In vitro studies showed that TMAO was able to raise the differentiation of mature adipocytes from preadipocytes, increase expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) from macrophages and neurons and reduce expression of tight junction proteins in blood brain barrier cells. Moreover, TMAO was able to activate microglia and raise the expression of the pro-inflammatory marker CD16/CD32, increasing aberrantly myelin phagocytosis.

SAMP8 a senescence accelerated mouse model was used to study the effect of aging (the main risk factor for AD) in peripheral inflammation and neuroinflammation. Studies were performed at three different ages: 2-months-old SAMP8 (young mice), 6-months-old SAMP8 (adult mice) and 10-month-old SAMP8 (old mice). Aging altered peripheral insulin sensitivity and glucose homeostasis in SAMP8 mice but not brain insulin signaling. Moreover, aging induced cognitive deficiencies and promoted peripheral and central inflammation. Finally, aging also induced gut dysbiosis, showing reduced diversity and changes in gut microbiota composition with enterotypes that could be associated to higher TMAO levels. This fact was further contrasted using LC-SM/SM what showed greater TMAO levels in serum of SAMP8 mice. Brain TMAO levels also increased with age in mice and humans.

Treatment with 3,3-dimethyl-1-butanol (DMB), which is a choline TMA lyase enzyme inhibitor that decreases TMAO serum levels, restored peripheral inflammation reducing fat adipose tissue and reversing insulin and glucose alterations. Moreover, DMB also

restored neuroinflammation decreasing expression of pro-inflammatory cytokines in hippocampus, reducing gliosis and restoring GFAP levels back to normal. Finally, SAMP8 performance in behavioral test was improved after DMB treatment ameliorating and restoring cognitive dysfunction.

INTRODUCTION

INTRODUCTION

1. THE GUT-BRAIN AXIS AS A LINK BETWEEN INSULIN RESISTANCE/OBESITY AND COGNITIVE DYSFUNCTION

Obesity and insulin resistance are two of the main epidemic problems that have a devastating impact on sustainable health of individuals on the long-term (1,2). According to literature, insulin resistance and obese individuals have an increased risk of developing neurodegenerative diseases on the long-term (3). For instance, clinical and experimental evidence indicates that obesity and/or metabolic alterations are linked to deficits in memory, learning, and executive functioning (4–6), and potentially brain atrophy (7,8). Furthermore, accumulating evidence indicates that diabetes and obesity during mid-life raises the risk of dementias like Alzheimer's disease (AD) later in life (9–11).

1.1 Gut microbiota

While much progress has been made in clarifying the neurobiological mechanisms underlying obesity/insulin resistance-associated cognitive impairment (12), only a few reports have studied the gut microbiome (13). The microbiome is shaped by host factors, like nutrients and genetics, but in turn is able to influence host biology in health and disease.

The gut microbiome has played a key role in the bidirectional gut-brain axis that integrates the gut and central nervous system (CNS) activities, and thus the concept of microbiome-gut-brain axis is emerging. A number of trials are revealing the way different forms of neuropsychiatric and neuroimmune disorders are related to or modulated by changes in the microbiome, microbiota-derived products and exogenous antibiotics and/or probiotics. Nowadays, it is thought that, through this gut-brain pathway, microbiota is able to affect brain plasticity and cognitive function.

1.1.1 Communication between gut microbiome and central nervous system

The connection between CNS and the gastro-intestinal (GI) tract is widely proven and it is essential in the modulation of gut function and homeostasis. Aside from research into the role of the gut-brain axis on these functions, there has been special attention on how these interactions could affect mental health (14). Various studies in animal models have established that in extreme conditions such as germ free (GF) or when altered under antibiotic treatment, gut microbiota correlates with changes in several neurotrophins and monoamine neurotransmitters involved in brain development and plasticity (15). Changes in brain chemistry in GF mice include a decrease in the *N*-methyl-d-aspartate (NMDA) receptor subunit *NR2B* mRNA expression, up-regulation of brain-derived neurotrophic factor (*BDNF*) and decreased expression of the serotonin receptor 1A (*5HT1A*) in the hippocampus (16). These findings show that the presence of commensal bacteria in the gut is crucial for normal brain development and increase the possibility that bacteria might influence brain plasticity and cognition later in life.

It has been proposed that the pathways of age-related gut dysbiosis and neurological decline are associated through the induction by the former of a chronic low-grade inflammation as a common basis for a broad spectrum of age-related pathologies (17). There are multiple ways in which gut dysbiosis could contribute to cognitive decline, including direct inflammatory stimulation, the production of proinflamamtory metabolites, and the loss of immune-regulatory function. Remarkably, dysbiosis-associated inflammation is strongly implicated in obesity and diabetes too. Both, obesity and diabetes, have been shown to exacerbate normal cognitive decline (16).

1.1.2 Diet induced changes in gut microbiota may induce cognitive alterations

Nutrition and dietary intake are one of the main factors that influence microbial diversity within the gut (18). There are significant shifts in gut microbiome composition according to differing diets (18,19). For instance, the Western-type diet i.e. high-fat (HFD), high-sugar (HFHS), or high polysaccharide-containing plant diets have been shown to significantly alter gut microbiome composition (18,19). Moreover, several epidemiological studies in elderly subjects have shown links between diet and cognitive function (20). Similar diet-related changes in cognitive flexibility have been found in mice fed either a high sucrose or HFD, possibly secondary to a shift in gut microbiota composition (20).

1.2 Inflammation: a possible link between insulin resistance/obesity and Alzheimer's disease

1.2.1 Systemic inflammation

Nowadays, obesity and insulin resistance are considered not only as a metabolic disorder, but also as an inflammatory disease affecting both innate and acquired immune system function (21,22). Certainly, obese and diabetic individuals frequently display basal low-grade systemic inflammation prompted by both adipose tissue (23) and gut microbiota (24), and increased susceptibility to immune-mediated diseases (25). Dietary factors such as some fatty acids lead to stimulation of the Toll-like receptor 4 (TLR4) on immune cells, and initiation of an inflammatory cascade (26). HFD is also associated with infiltration of macrophages into white adipose tissue (WAT), apoptosis of adipocytes, and decreased WAT vascularity (26,27). This macrophage proliferation, coupled with increased TLR4 and other pattern recognition receptors on adipocytes, leads to a raise in proinflamamtory cytokines (26,28,29), such as tumor necrosis factor alpha (TNF-α), feeding-related peptides (leptin and resistin), plasminogen activator inhibitor 1, C-reactive protein and interleukins such as IL-1β and IL-6 (30).

1.2.2 Central inflammation

Sometime ago, the systemic inflammation associated with obesity and insulin resistance was identified (31) but the proposal that obesity could also result in central inflammation is relatively recent. Interestingly, clinical studies have reported positive associations in obese/diabetic patients between peripheral inflammatory status and cognitive decline or mood symptoms (32). On the other hand, surgery-induced weight loss is associated with reduced peripheral inflammation (23) and significant

improvement in emotional status (33,34). Moreover, studies performed in rodent models of obesity show that inflammation occurs within the brain too, particularly in areas involved in mood regulation and memory formation like the hippocampus or the cortex (35).

Peripheral cytokines are able to act on the brain and induce local production of cytokines (36). Accordingly, central inflammation is observed after HFD and in genetic models of obesity (37). Interestingly, the hippocampus and the cortex, regions implicated in cognitive processing, learning and memory, could be particularly vulnerable to inflammation in obesity. For example, elevated TNF-α and IBA1 (microglial marker) levels have been described in this region after 20 weeks of HFD (38). The astrocyte markers GFAP and APP, an indicator of AD-like pathology, are also augmented in those areas after long-term HFD and peripheral insulin resistance (39,40). In this context, it has been suggested that obese patients show a dysfunction in adipocytes with the subsequent changes in its secretory activity that could lead to a greater production of pro-inflammatory cytokines that could cross the blood brain barrier (BBB) and induce neuroinflammation, central insulin resistance and decease cognitive function (41,42).

Taking into account these data, it could be concluded that obesity and insulin resistance lead to systemic and central inflammation, with increased circulating proinflamamtory cytokines that have been associated to impaired cognitive function (21,43). Thus, changes in gut microbiota may have taken place first and triggered an inflammatory pathway from the gut to the brain. Gut microbiota is able to synthesize a range of compounds that could mediate local and systemic inflammation. Therefore, gut microbiota dysbiosis may be involved in the pathophysiology of insulin resistance/obesity and cognitive decline through its impact on local and systemic inflammation. However, which is the gut microbiota-derived compound that mediates the local and systemic inflammation?

2. TRIMETHYLAMINE N-OXIDE (TMAO)

Choline, a trimethylamine-containing compound and part of the head group of phosphatidylcholine, is metabolized by gut microbiota to produce an intermediate compound known as trimethylamine (TMA). TMA is rapidly further oxidized by hepatic flavin monooxygenases to form TMAO. At first TMAO was thought to be a waste product of choline metabolism without action in our organism but in 2011, Wang et al. (44) proposed a potential role of a complex phosphatidylcholine–choline metabolic pathway involving gut microbiota in contributing to the pathogenesis of atherosclerosis and nowadays there is convincing evidence suggesting an association between TMAO and inflammation (45–49). However, the exact mechanism underlying this correlation is still unknown.

2.1 TMAO metabolism

TMAO is mainly generated in the liver from the oxidation of TMA which is formed in the gut by the action of microbiota from substrates such us betaine, L-carnitine and its metabolite γ-butyrobetaine (GBB), choline and other choline-containing compounds which are present in the diet. There are numerous enzymes involved in TMA formation (50) (Figure 1).

TMAO and TMA can be found in a natural way in some foods like fish. Approximately half of the ingested TMAO is absorbed unchanged and then excreted in urine. The remaining 50% is converted into TMA in the gut by the action of TMAO reductase (51). However, TMA can be oxidized to TMAO again by the action of TMA monooxigenase, which is present in some gut microorganisms (52,53). Furthermore, some bacteria are able to deplete TMA and TMAO to form dimethylamine (DMA) and formaldehyde (HCHO) by the action of the enzymes trimethylamine dehydrogenase (TMADH) and TMAO demethylase (52).

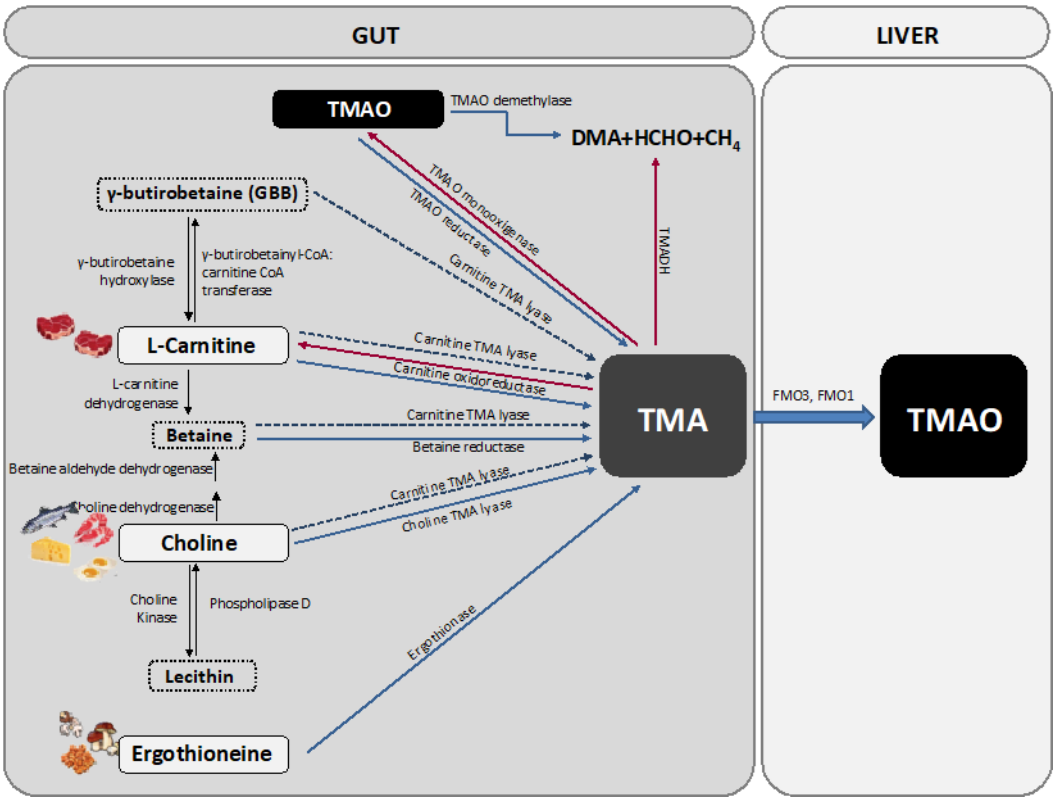


Figure 1. Pathways for (TMAO) synthesis. Trimethylamine (TMA) is formed in the intestinal lumen when gut microbiota metabolize carnitine, choline, and choline-containing compounds in the diet. TMA can be absorbed from the intestine. This absorbed TMA is delivered to the liver where flavin-dependent monooxygenase (FMO) isoforms 1 and 3 convert it to TMAO. DMA: dimethylamine; HCHO: formaldhyde; TMADH: trimethylamine dehydrogenase; CoA: coenzyme A.

The two main sources of TMA are choline and L-carnitine. Choline can be found in many foods as free choline or as a part of several compounds (phosphocholine, phosphatidylcholine, sphingomyelin, etc.). Most of these sources have an animal origin. Lecithin also known as phosphatidylcholine is one of the main choline-containing

compounds and can be converted into choline by the enzyme Phospholipase D. Interconversion of choline and lecithin is bidirectional, being the conversion of choline to lecithin catalyzed by the enzyme choline kinase (51). Moreover, choline can be transformed into TMA by the action of the enzyme choline TMA lyase, or be transformed into betaine by the sequential action of two enzymes (choline dehydrogenase and betaine aldehyde dehydrogenase) (51) (Figure 1).

Additionally, betaine, which is mostly found in plants, can be reduced to TMA by betaine reductase in a coupled reduction-oxidation reaction where it acts as an electron acceptor. Alternatively, betaine plays an important role as a methyl donor in the betaine homocysteine methyltransferase pathway too.

Apart from choline, L-carnitine is the other main precursor of TMA. The main enzyme responsible for the conversion of L-carnitine into TMA is carnitine oxidoreductase (54) (Figure 1). However, there is another enzyme known as Carnitine TMA lyase with substrate promiscuity for carnitine, choline, betaine and GBB. Carnitine can be only found in foods as its L-stereoisomer, with animal products containing the biggest amounts (51,55). L-carnitine can also be transformed into two other precursors, i.e., betaine by the action of L-carnitine dehydrogenase and GBB by the enzyme γ-butyrobetainyl-CoA:carnitine CoA transferase. Alternatively, GBB can be reconverted into L-carnitine by the enzyme γ-butyrobetaine hydroxylase or be transformed into TMA by the action of the previous cited enzyme carnitine TMA lyase (51).

Finally, another source of TMA is ergothioneine, a biogenic amine (a derivative of histidine) that can be obtained from some dietary sources such as mushrooms, meat products (mainly kidney and liver) and several types of beans. Ergothioneine is degraded by the enzyme ergothionase to produce TMA (51).

Most of TMA ingested or formed in the gut is rapidly absorbed into the portal circulation by passive diffusion and then oxidized to TMAO by the action of hepatic flavin containing monooxygenases FMO3 and FMO1 (Figure 1). FMO1 has tenfold lower specific activity in the liver than does FMO3, so FMO3 is the main enzyme responsible for the conversion of TMA into TMAO (55).

2.2 Importance of microbiota in TMA production

Microbiota plays a crucial role in TMA formation. Specific microbiota is needed for the conversion of the substrates previously cited into TMA as seen in some studies using gnotobiotic mice (56,57). Moreover, some studies performed with antibiotics in rats and humans have revealed that the production of TMA and TMAO is almost totally suppressed with the use of broad-spectrum antibiotics like Ciprofloxacin, Vancomycin or Metronidazole. However, TMAO levels return to normal after one month of the withdrawal of the antibiotics (44,58).

Some of the bacteria involved in TMA formation belong to the phyla Firmicutes (*Sporosarcina*), Actinobacteria and Proteobacteria, especially those bacteria belonging to the class Gammaproteobacteria (*E. coli, Citrobacter, Klebsiella pneumoniae, Providencia, and Shigella*), and Betaproteobacteria (*Achromobacter*). However, it seems that bacteria belonging to the phylum Bacteroidetes are not able to produce TMA from the dietary compounds (59). Because of that, the Firmicutes/Bacteroidetes ratio has been recently used to predict and study TMAO concentrations in plasma (60,61).

Genes *CutC* and *CutD* are indispensable to transform choline into TMA for *Desulfovibrio alaskensis* and *Desulfovibrio desulfuricans* (55). On the other hand, other studies have revealed that *CntA* and *CntB* gens encode the two subunits of the oxidoreductase enzyme necessary to convert L-carnitine into TMA in some bacteria of the gender *Acinetobacter* and *Serratia* (55). Moreover, *YeaW/YeaX* are another gene pair that encode some oxygenase and oxidoreductase enzymes with substrate promiscuity for choline, betaine, carnitine and GBB (55). These genes, in addition to the orthologs and homologs of the *CntA/CntB* and *YeaW/YeaX* gene pairs, can be found in a wide range of bacteria present in the gut.

2.3 TMAO distribution and excretion

Despite dietary TMAO has been purported to enter the same metabolic pathway as its putative precursors, its metabolism in humans is poorly understood. To quantitatively elucidate the metabolic fate of orally consumed TMAO, a recent study traced the metabolic fate of orally consumed TMAO in humans using a stable isotope approach (62). They reported that orally consumed TMAO is largely absorbed and does not require microbial or hepatic processing, has a high turnover and rapid clearance rate and is taken up by extrahepatic tissues.

Alternatively, as mentioned before, most of TMA is absorbed by passive diffusion across the enterocyte membranes. Some of the TMA ingested and formed in the gut can be eliminated in feces, but the amount of TMA excreted in feces has not been carefully quantified. Once absorbed, nearly 95% of TMA is oxidized and it is afterwards excreted in urine in a 3:95 TMA:TMAO ratio within 24 hours, only 4% is excreted in feces and less than 1% is eliminated in the breath (55).

Finally, TMAO can also be metabolized to DMA, HCHO, ammonia and methane by some methanogenic bacteria containing the enzyme TMAO demethylase (52).

2.4 TMAO detection and measurement

In most studies, TMAO is measured in urine and plasma samples although it is sometimes measured in serum samples too. The methods that are often used include liquid chromatography mass spectrometry (LC-MS), mainly stable isotope dilution high performance liquid chromatography with electrospray ionization tandem mass spectrometry (DIS-HPLC-SM/SM), proton nuclear magnetic resonance spectrometry (1H-NMR), headspace gas chromatography (GC) and matrix-assisted laser

desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Other technique less common is Fast Atom Bombardment- mass spectrometry (FAB-SM).

It has been recently described a new method to measure TMAO in urine combining TMA derivation with ethyl-bromoacetate and liquid chromatography with single ion monitoring (LC-SIM) (63).

Furthermore, in some studies TMAO has been measured in liver (64) and fecal waters (65) using 1H-NMR but it has also been measured in other tissues such as brain, muscle, kidney or intestine using the method of reduction from TMAO to TMA with a mixture of ferrous sulphate and EDTA as described by Wekell and Barnett (1991) (66) or a modification of their method (67,68). Additionally, in a recent study, TMAO was detected and measured in cerebrospinal fluid (CSF) using LC-MS (69).

2.5 Factors that influence TMAO levels

Plasma TMAO levels show wide inter- and intra-individual variations. These levels are influenced by several factors (70). One of the main factors influencing TMAO levels is aging. Some studies performed in human and rats have revealed that plasma TMAO levels are closely related to aging showing increasing levels with age (71,72).

Apart from aging, diet seems to be the other major factor that plays a key role in TMAO formation (Table 1). For example, vegetables of the family *Brassicaceae* can reduce FMO3 activity. Moreover, vegetarians and people consuming diets based mainly in plants are less able to produce TMA from L-carnitine than omnivorous people (73).

On the other hand, HFD and Western-like diets have been related to increased plasma TMAO levels (45,74–76). However, other studies have showed no changes in plasma TMAO levels after a HFD (77) or have even suggested that fats may suppress TMAO formation (78). Moreover, TMAO changes in plasma levels seem to be postprandial and not in fasting plasma TMAO levels, as fasting may reduce plasma TMAO levels (74,79).

Proteins content also seems to have a high positive correlation with TMAO excretion in urine (80). A possible explanation for this finding is that TMAO may be synthetized to arrange the excess of amine groups (81). Furthermore, a low protein diet in patients with chronic kidney disease (CKD) resulted in lower plasma TMAO levels (82).

Additionally, some studies suggest that diets with high content in non-digestible carbohydrates could reduce TMAO formation by remodeling gut microbiota (65) while other studies reports the opposite effect, suggesting that diets high in non-digestible starch would increase plasma TMAO levels in the short term (83).

Vitamin D also seems to play a role in plasma TMAO levels reducing them (84). In addition, diets with pistachio supplementation are also suggested to reduce TMAO formation (85) while histidine supplementation have been suggested to increase plasma and urine TMAO levels (86). Other studies have showed that high plasma TMAO

concentrations may reflect a specific metabolic pattern characterized by low HDL and phospholipids and hypomethylation (86,87).

FMO3 expression also plays an important role in TMAO levels. Firstly, cholic acid (a bile salt) can induce FMO3 expression via the bile acid–activated nuclear receptor FXR, thereby increasing plasma TMAO levels. On the other hand, in male mice FMO3 has been found to be highly induced by diabetes. Sex hormones are also important for TMAO levels as estrogens may act as inductors of FMO3, while testosterone acts as a suppressor so levels tend to be higher in woman. Additionally, TMAO levels decrease at the onset and during menstruation causing an accumulation of TMA also known as trimethylaminuria (55).

Finally, renal clearance may play a major and critical role in plasma TMAO levels. Emerging studies have suggested that many of the studies that have reported high TMAO levels are confounded by impaired kidney function. Among them, Mueller et al., (88) did an 8 years follow-up study trying to relate plasma TMAO, betaine or choline levels with cardiovascular disease (CVD), but the results showed that the levels were confounded by impaired kidney function or poor metabolic control. Other studies have revealed that TMAO levels are increased in renal insufficiency but concentrations normalize after renal transplantation (89). There seems to be an inverse correlation between TMAO levels and glomerular filtration rate (GFR); levels are higher the more the GFR is reduced (90). However, there are also some studies suggesting that TMAO can contribute to the development of renal insufficiency (91) and/or serve as a predictor of glomerular injury (92). Remarkably, patients with type 2 diabetes mellitus (T2DM) and chronic kidney disease have showed a higher proportion of TMA-producing microbiota and a positive correlation between TMAO and some biomarkers of inflammation and endothelial dysfunction was found (93) .

Table 1. Effects of different types of diet on gut microbiota and TMAO levels in different experimental models and metabolic circumstances

Type of diet	Influence on microbiota and TMAO	Consequences and Remarks	References
High fat diet (mice)	↑ plasma TMAO	Obesity and metabolic problems (not prevented with the use of DMB) Renal fibrosis, oxidative stress and inflammation of the kidney (prevented with DMB)	(76)
High fat diet enriched in phosphatydilcholine (rats)	↑TMAO in plasma and liver	Hyperlipidemia. TMAO levels decrease and lecithin levels increase with treatment of *Gynostemma pentaphyllum*, but not with treatment of atorvastatin.	(94)
High fat diet (human)	↑ postpandrial plasma TMAO levels, but not fasting ones.	In the short term, a reduction of plasma TMAO clearance is observed	(74)
High fat diet (human)	↑ plasma TMAO	The increase of TMAO levels is not prevented with the use of probiotics (VSL #3), though there is less weight gain and fat. The magnitude of the change in the levels of TMAO is correlated with systolic pressure and carotid pulse	(95)
High fat diet	↑Firmicutes and Proteobacteria ↓Bacteroidetes	More production of TMAO	(61)
Low fat diet	↑Bacteroidetes and ↓Firmicutes	Lower production of TMAO	(61)
High fat diet enriched in fish oils (FO) (mice)	↑TMAO plasma	FO improve the adverse effects produced by TMAO (tolerance to glucose and adipose tissue inflammation)	(96)
High protein diet	↑TMAO orina	High correlation with daily nitrogen excreted through the urinary tract	(80)
Low protein diet	↓TMAO plasma	A diet low in proteins in patients with CKD resulted in lower plasma TMAO levels	(82)
Similar to Western (mice)	↑TMAO plasma	Obesity and Dyslipidemia (not prevented with the use of DMB) Cardiac dysfunction and fibrosis of heart with increased expression of Proinflamamtory Cytokines, tumor necrosis factor and interleukin IL-1β and reduced expression of anti-inflammatory cytokines (IL-10) (prevented with DMB)	(45)
Rich in indigestible CH (human)	↓production of TMAO Gut microbiota alterations	Significant weight loss in children with simple obesity or Prader Willi Syndrome (PWS). Better state of inflammation.	(65)
Rich in CH and/or low in CH and rich in indigestible starch (human)	↑TMAO plasma	The diet does not improve short-term biomarkers of CVR. It mitigates the postprandial glucose and insulin response to hearty meals.	(83)
Supplementation with pistachios (human)	↓production of TMAO	Improvement of metabolic disorders associated with insulin resistance and T2DM.	(85)
Supplementation with histidine (human)	↑TMAO en plasma and urine.	Lower production of lipids and glucose	(86)
Vegetarian (human)	Changes in gut microbiota	Vegetables of the family Cruciferae can reduce FMO3 activity Reduced ability to produce TMA from L-carnitine.	(73)

CKD: Chronic kidney disease; CVR: cardiovascular risk; DMB: 3,3-Dimethyl-1-butanol; T2DM: type II Diabetes mellitus; FO: fish oil; His: histamine; TMAO: Trimethylamine N-oxide

3. TMAO AND NEURODEGENERATIVE DISEASES

The gut-brain axis has recently started to gain importance. This crosstalk involves the participation of the autonomic, neuroendocrine, and immune systems together with metabolites and neuromodulatory molecules produced and released by gut microbiota (97) which is composed of more than 100 trillion bacteria in adults, nearly ten times as many as cells in the body, containing more than thousands different species of bacteria (98).

The gut microbiota differs from one individual to another, conferring each one personal identity (99). Based on the data obtained by several studies, a healthy microbiota is the one that maintains community stability and species diversity, defining a good microbiota, so there seems to be a connection between the gut microbiome and some neurological disorders.

In this context, TMAO has been recently described like a metabolite produced by gut microbiota and metabolized in the liver that plays a role in systemic inflammation, atherosclerosis, T2DM and vascular dysfunction, but it has also been suggested to reach the brain and be involved in neuropathologies (50).

It has been controversial if TMAO could reach the brain, but recent studies have reported TMAO levels in CSF (69,100–102). However, it is not fully known if it crosses the BBB or if it is synthetized in the brain as FMO3 is also expressed (although to a much lower extent) in brain (103,104). Nevertheless, there are some studies in human microphysiological systems (105) and animals (106) that suggest TMAO crosses BBB.

Additionally, some recent studies showed a positive correlation between plasma TMAO levels and CSF TMAO levels (101,102), thus suggesting TMAO in CSF originates mainly from blood and it could cross BBB and/or blood-CSF barrier by passive diffusion. However, it seems that TMAO could easier cross a disrupted BBB. Indeed, a recent study of 290 patients showed that healthy controls had significantly lower CSF TMAO levels than patients with blood-CSF barrier dysfunction (measured by CSF/serum albumin quotient) (101).

Furthermore, TMAO has been suggested to cause BBB disruption by reducing the expression of tight junction proteins, such as claudin-5 and zonulin (ZO-1), favoring its access to the brain (107,108).

The role of TMAO in brain and neurodegenerative diseases is not fully understood as it could have both protective and harmful effects (Table 2). Some studies have reported that TMAO may exert protective effects acting as a natural osmolyte, reducing the stress in endoplasmic reticulum (ER) and returning the primary structure to misfolding proteins. Chaperones like TMAO supports enzymatic activity preventing protein degeneration from changes in pH or osmolyte concentrations (109).

Organic osmolytes like TMAO could exert protective effects in prion diseases. For example, TMAO inhibits the conversion of the scrapie prion protein (PrPC) into its pathogenic isoform (PrPSc), which is associated with transmissible spongiform encephalopathies. TMAO seems not to affect the current population of PrPSc, but interferes with the formation of PrPSc from newly sinthethized PrPC (110).

The protective effect exerted by TMAO as a chemical chaperone has also been observed in other diseases, such as Machado-Joseph disease/spinocerebellar ataxia-3 (MJD/SCA-3) (111) and amyotrophic lateral sclerosis (112). MJD/SCA-3 is an inherited neurodegenerative disorder where the truncated form of mutated ataxin-3 causes aggregation and cell death in vitro and in vivo. TMAO was found to reduce aggregate formation, cell death and cytotoxicity induced by truncated expanded ataxin-3 (111).

However, the major limitation in using chemical chaperones like TMAO as drugs is their very active concentration, in the millimolar range. Because of that, Getter et al. (112) designed a lipophilic derivative of TMAO, which was able to improve neurological functions in mice, preventing ER-stress-induced apoptosis of NSC-34 motor neuron-like cells and primary mouse astrocytes.

Finally, TMAO has been also shown to play a role in the conformation of intrinsically disordered proteins (IDP). IDP are proteins that do not have a stable three-dimensional structure. One of these IDP is α-synuclein, which can aggregate into toxic protofibrils, is one of the main components of Lewy bodies and has been linked to Parkinson disease (PD) and other neurodegenerative diseases (113). TMAO suppresses the formation of extended conformations and can act as a protecting osmolyte leading to compact and folded forms of α-synuclein. This effect could probably prevent the α-synuclein aggregation and formation of insoluble fibrils that cause PD (113). When the concentration of TMAO is high enough, α-synuclein forms oligomers in which the subunits are folded and are not able to fibrillate (114). Interestingly, a study performed in 18 subjects with PD showed CSF TMAO levels were elevated in PD patients with motor fluctuation (102).

Table 2. Relationships between neurodegenerative disorders and TMAO in different experimental conditions

Neurodegenerative disease	Remarks about TMAO	References
AD	Share common genetic pathways	(115)
AD	No relationship, associations are due to confounding	(116)
AD	TMAO stimulates tau-induced tubulin assembly	(117)
AD	TMAO can promote and enhance the assembly of microtubules in mutant and hyperphosphorylated Tau	(118)
AD	TMAO restores mutant Tau activity reducing the critical concentration of tubulin necessary for assembly	(119)
AD	TMAO is able to stabilize and modify the aggregation of the amyloid beta (Aβ) peptide, thus favoring the formation of plaques	(120)
AD, non-AD related dementia and other neurological disorders	No relation between CSF TMAO levels and neurological diagnoses	(69)
AD	Greater CSF TMAO levels in AD and MCI patients compared to HC. No differences between AD and MCI TMAO levels. CSF TMAO levels were associated to several AD biomarkers like phosphorylated tau (p-Tau), total tau (t-Tau), p-Tau/Aβ42 and neurofilament light chain protein.	(100)
PD	CSF TMAO levels are elevated in PD patients with motor fluctuation	(102)
PD	Low plasma TMAO levels are associated with faster increases in Levodopa equivalent dose and tend to increase the risk for PD-dementia conversion	(121)
PD	TMAO leads to compact and folded forms of α-synuclein, what could prevent α-synuclein aggregation and formation of insoluble fibrils that cause PD	(113,122)
Blood-CSF barrier dysfunction	Greater CSF levels in patients with blood-CSF barrier dysfunction	(101)
ALS	A lipophilic derivative of TMAO was able to improve neurological functions in mice, preventing ER-stress-induced apoptosis	(112)
MJD/SCA-3	TMAO reduced aggregate formation, cell death and cytotoxicity induced by truncated expanded ataxin-3	(111)
Transmissible spongiform encephalopathies	TMAO inhibits the conversion of the scrapie prion protein (PrPC) into its pathogenic isoform.	(110).

3.1 TMAO and Alzheimer's disease

TMAO has been recently associated with several neuropathologies including AD. Some studies report TMAO is closely related to AD due to common genetic pathways underlying AD biomarkers (115). However, a recent bidirectional Mendelian randomization study stablished that TMAO was not related to AD and the associations observed in other studies might be due to confounding (116).

If TMAO is beneficial or harmful for AD remains elusive. On one hand, TMAO may be beneficial for AD as it can stimulate tau-induced tubulin assembly (117). Moreover, later studies have showed that TMAO can promote and enhance the assembly of microtubules in mutant and hyperphosphorylated Tau protein, reaching in the majority of cases a greater protein efficiency ratio than in wild-type Tau (118). TMAO seems not to act by dephosphorylating tau protein; it facilitates the binding between tau protein and tubulin by reducing the critical concentration of tubulin necessary for assembly (119).

On the other hand, TMAO may be harmful for AD due to its interactions with amyloid beta (Aβ) peptides. It has been previously exposed that TMAO acts as an osmolyte stabilizing the proteins (see section 3). Nevertheless, in some cases, overstabilization results in stiffer molecules that tend to form aggregates. Indeed, some studies showed that TMAO is able to stabilize and modify the aggregation of the Aβ peptide (120), favoring and accelerating the transformation of the random string of the Aβ peptide to its β-conformation and stabilizing the resulting protofibrils, which can originate fibers that tend to aggregate and form plaques.

Regarding CSF TMAO levels, whether higher levels are present in AD patients remains unknown. The study of Del Rio et al., 2017 (69) was the first reporting TMAO levels in CSF of 58 patients, but they found no relation between TMAO levels and neurological diagnoses (AD, non-AD related dementia and other neurological disorders). However, another study measuring CSF TMAO levels in 410 patients (AD, mild cognitive impairment (MCI) and healthy controls (HC)) (100), reported higher CSF TMAO levels in AD and MCI patients compared to HC, without showing differences between AD and MCI patients. In the same study, higher CSF TMAO levels were associated to several AD biomarkers like phosphorylated tau (p-Tau), total tau (t-Tau), p-Tau/Aβ42 and neurofilament light chain protein. Other AD biomarkers such us Aβ42/Aβ40 or neurogranin were not associated to higher CSF TMAO levels (100).

3.2 Changes in TMAO levels induced by risk factors in Alzheimer's disease

As already mentioned, the most important factor that impact and modulate gut microbiome are dietary changes (123). In addition, many of the factors that can modulate the composition of gut microbiota, such as ageing, stress or obesity, are also considered risk factors for AD.

Gut microbiota profile may shift in some pathological circumstances such as depression (124) or neurodegenerative diseases (125,126). However, this issue raises an important question: is the disease *per se* who changes the microbiota or are the disease risk factors the ones affecting gut microbiome? Negative lifestyle aspects, among people living in our modern societies, are considered important risk factors for the development of AD (127). The most striking result of epidemiological studies is that substantial increase of AD in developing countries is associated with changes in national diets (128). Furthermore, apart from the main AD risk factor that is aging, there are many undesirable lifestyle factors in the modern society that may contribute to AD development. These factors include unhealthy diet and stress, and, in turn, gut microbiome is highly sensitive to these factors. From this point of view, studying the links between aging, modern lifestyle, gut microbiome and AD is an important task that requires special attention.

Since advanced age is a major risk factor for AD, age-related physiological changes, including changes in the microbiome, may play a certain role in the development of dementia. In this regard, a number of studies have shown that the composition of the gut microbiome undergoes significant changes with age (129,130).

Several studies show that gut composition differs between old and young people. However, it is not possible to set a specific point where the microbiome gets altered as it occur gradually through time (131,132).

Colonization of the gut may start in utero from the placenta. Most of infant microbiome is acquired during birth and follows with feeding and exposure to environment (97). Gut microbiota evolves rapidly the first three years and at the age of 3 years old, microbiota composition becomes similar to adults (97,131). Childhood and adolescence are two critical periods for gut microbiota composition and neuronal development (133) and get more diverse with dietary changes.

Aging is closely related with reduced microbiota diversity and this fact has been related to frailty while healthy aging is associated with diverse microbiome (133). Several authors have observed that the levels of *Bifidobacterium* and *Lactobacillus* are lower in elderly people compared to young individuals (134,135). One of the possible explanations for the reduced number of species and quantitative composition of bacteria in the elderly people is the decrease in their adhesion to the intestinal wall due to changes in the chemical composition and structure of the colon mucous membrane, causing restricted functionality and immunological reactivity in the intestine as well as increased susceptibility to gastrointestinal infections (136). Moreover, many authors have noted that *Bacteroides* species diversity changes with age (137,138) and the growth of proteolytic bacteria, such as *Fusobacteria*, *Propionibacteria*, and *Clostridia*, has been observed in the intestinal microbiota of elderly people leading to the development of putrefactive processes. Also, an increased number of proinflamamtory enterobacteria, streptococci, staphylococci, and yeast cells have been found which may be associated with an elevated level of serum antibodies to commensal (normal)

intestinal microbiota. It should be noticed that the impact of aging over microbiome is intimately associated with diet and lifestyle so it remains uncertain if aging per se, independent of external influences is able to alter microbiome (97). For instance, while bacterial cells do not age per se, people getting older start to take antibiotics, other drugs and begin to present comorbidities affecting gut.

Aging induced-dysbiosis is able to reduce the expression of tight junction proteins in the gut, producing intestinal inflammation and a major permeability, what favours the passage of gut metabolites to the circulatory system (139). Among these microbial metabolites, TMAO has been recently shown to be related to inflammation and cognitive decline (50).

As mentioned before, TMAO levels depend on several factors but they are closely related to aging. Some studies performed in human and rats have revealed that plasma TMAO levels increase with age (71,140) and other report that the association between aging and TMAO is much stronger than the link between TMAO and other factors which is limited and inconsistent (141).

3.3 TMAO and aging: studies in SAMP8 mouse model

The senescence-accelerated mouse prone 8 (SAMP8) is a mouse model which is often used to study the effects of aging and age-related neurodegenerative diseases like AD as it present irreversible senescence and share many phenotypical characteristic with AD patients like age-related memory and learning deficits, neuroinflammation and oxidative stress. This model also shows other important AD hallmarks such as Tau hyperphosphorylation and Aβ accumulation (142,143).

SAMP8 model was developed by professor Takeda by selective inbreeding of the AKR/J strain. Some of those mice presented features of early senescence like loss of activity, alopecia, shortened life-span… Those litters were selected as progenitors of the senescence-prone series and the other as progenitor of the senescence resistant strains. Eleven senescence-prone strains and seven senescence-resistant subtypes were obtained, being Senescence Accelerated Mouse-Resistant 1 (SAMR1) a strain with normal pattern of aging and the one considered as the control reference in most studies using SAMP8 model (142).

Life spam of the senescence-resistant strains ranges from 19 to 21 months while life spam of the senescence-accelerated series ranges from 10 to 17 months. The progressive cognitive decline and neurodegeneration have led to consider SAMP8 mice as a good model for sporadic AD (144) with several benefits over gene-modified models as it is a model based on age rather on mutations and it could depict a better understanding of age-related events and represent the multifactorial etiology of AD (145–147).

As mentioned before, SAMP8 model show several AD hallmarks like oxidative stress and impaired antioxidant defense (148), BBB dysfunction (149), neuroinflammation

(150), Tau pathology (151) and Aβ deposition (151–153). However, these deposits of Aβ are clustered granules and don't form plaque-like structures (152,153).

Oxidative stress is the earliest manifestation of SAMP8 model and starts at 2 months of age, cognitive decline, neuroinflammation and Tau hyperphosphorylation appear at 5-6 months of age and increased soluble Aβ as well as gliosis occurs at 8 months (145). Moreover, in hippocampus BBB integrity and permeability has been shown to be altered in SAMP8 at 4 months of age (154) (Figure 2).

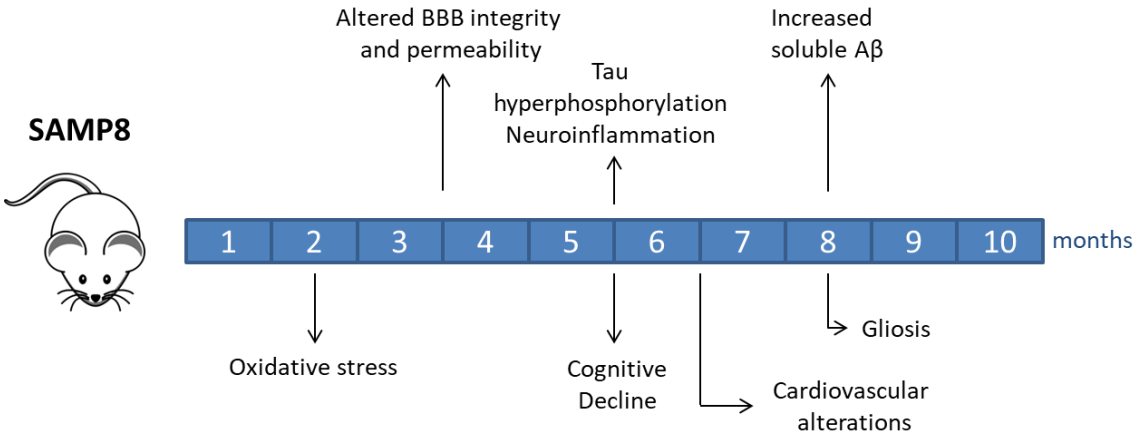


Figure 2. Phenotypical characteristics developed by SAMP8 mouse model. Schematic representation of the progression of the phenotypical characteristics in the senescence accelerated mouse prone-8 model (SAMP8). BBB: Blood Brain Barrier.

Previous studies have showed that plasma TMAO levels increase in SAMR1 at the age of 10 months and in SAMP8 at the age of 6 months (155). Nevertheless, there was no behavioral test that could measure the impact of this increase in cognition and neuroinflammation. Another study of the same research group showed that 6 months old SAMP8 mice had greater levels of plasma TMAO levels than SAMR1 age-matched and they presented impaired cognition as seen by the results of Morris Water Maze test (MWM) and Y maze test.

However, to our knowledge, no studies reporting brain TMAO levels in SAMP8 mice have been published.

4. UNDERLYING MECHANISM OF THE DELETERIOUS EFFECTS OF TMAO

4.1 Inflammation

Several studies have revealed an increase in the expression of proinflamamtory cytokines when plasma TMAO levels are elevated. The study performed by Rohrmann

et al. (2016) (47) described a link between low grade inflammation and plasma TMAO levels. When the concentration of plasma TMAO was augmented there was an overexpression of TNF-α, IL-6 and C-reactive protein.

The relationship between TMAO and inflammation seems pretty clear. For example, studies performed in fetal human colon cells found a dose- and time-dependent increase of oxidative stress when TMAO was added. Furthermore, they also observed a dose-dependent inhibition of the expression of ATG16L1, decreasing ATG16L1-induced autophagy and activating NLR family Pyrin domain containing 3 (NLRP3) inflammasome, which has been recently found to be critical for the development of atherosclerosis and has been also linked to AD (108,156). Those harmful effects were significantly reversed by siRNA-mediated knockdown of *NLRP3* and over-expression of *ATG16L1* (49).

Other experiments performed in Human Umbilical Vein Endothelial Cells (HUVEC) and mice also revealed a link between TMAO administration and inflammation by activation of NLRP3 inflammasome. One of these studies suggests that this activations occurs through the inhibition of SIRT3-SOD2-mitochondrial ROS signaling pathway. Additionally, TMAO could not further inhibit *Sod2* in SIRT3 siRNA-treated HUVEC and aortas from $SIRT3^{-/-}$ mice (46). Other studies propose that this activation is mediated through ROS-TXNIP pathway, elevating inflammatory cytokines, IL-1β and IL-18 and inhibiting endothelial nitric oxide synthase (eNOS) in a dose and time dependent manner, thus decreasing the production of nitric oxide (NO). These effects can be reversed with the use of N-acetylcysteine or siRNA-mediated knockdown of *TXNIP* and *NLRP3* (48).

Moreover, some trials performed in carotid artery endothelial cells revealed that TMAO significantly increases the activation and formation of NLRP3, caspase-1 activity, IL-1β production and cell permeability. This activation of NLRP3 was abolished with NLRP3 siRNA or caspase-1 inhibitor, WEHD (108).

Regarding neurodegenerative diseases, an emerging hypothesis is that the gut microbiota may influence neuroinflammation in AD. In particular, it has been proposed that in AD, the presence of dysbiosis, causing the breakdown of the intestinal permeability, can lead to an inflammatory condition not limited to the gut, since the pro-inflammatory cytokines can get into the bloodstream and reach the brain (157). The importance of inflammation should not be underestimated, since several evidences support its crucial role in several chronic disorders, such as AD (158).

In an elegant study, Minter et al. (159) investigated the effects of high-dose antibiotic cocktail on gut microbiota and the level of inflammation in APP/PS1 mice and found that the amount of circulating inflammatory cytokines released by gut microbes was modified by antibiotics. Furthermore, they assessed some of those cytokine levels in lymphocyte and splenic cells and concluded that there was no change in their levels, which reinforces the notion that the signal molecules generated by the gut microbiomes influence the brain homeostasis.

Previous studies have investigated the association between gut bacteria and AD and have demonstrated a connection between cognitive impairment, brain amyloidosis and the existence of inflammatory markers in circulation (160). Authors found that AD patients with cognitive decline had higher circulating levels of proinflamamtory cytokines (IL-6, CXCL2, NLRP3 and IL-1β) and lower levels of anti-inflammatory cytokine (IL-10). Interestingly, these cytokine level alterations correlated positively with *Escherichia*/*Shigella* amount. Consequently, they hypothesized that alterations of the gut composition, i.e., increased levels of proinflamamtory (*Escherichia*/*Shigella*) and reduced levels of anti-inflammatory (*Eubacterium rectale*) bacteria, may play a role in AD cognitive impairment (160).

An important issue is that many intestinal bacteria secrete lipopolysaccharide (LPS). LPS is the key component of gram-negative bacteria's outer cell membrane, which may induce neuro-inflammatory reactions in the case of penetration from the intestinal cavity into the bloodstream. Post-mortem studies performed in AD patients have shown that the level of LPS in the neocortex and hippocampus are two to three times higher than in older people of the same age without cognitive disorders (161). Moreover, intraventricular administration of LPS in mice for 4 weeks can cause chronic neuroinflammation, nerve cell death of the entorhinal cortex and synaptic plasticity impairment of the neurons of the dentate gyrus of the hippocampus (162).

Microglia, the brain resident macrophages, play an important role on neuro-inflammation. There is evidence that LPS secreted by bacteria can activate the proinflamamtory NFκB in human primary microglial cells (163), that is a transcription factor involved in the pathogenesis of AD. NFκB induces transcription of proinflamamtory miRNAs (miRNA-9, miRNA-34a, miRNA-125b, miRNA-146a, and miRNA-155) activating neuro-inflammatory mediators and inhibiting phagocytosis (163). Indeed, it has been shown that micro-RNA-34a inhibits TREM2 expression (the triggering receptor expressed on microglia/myeloid cells-2), thereby disrupting microglia phagocytic potential and increasing Aβ42 accumulation (164).

Inflammatory mediators enter CNS via the circulatory system as well as the lymphatic system linking the digestive system and CNS (165,166). It has been shown that in the 5xFAD AD mouse model the number of T helper (Th) $CD4^+$ is significantly higher in gut-associated lymphoid tissues (GALT) (166). Besides this phenotypic characterization, the authors observed that the concentration of IL-17 per cell in transgenic mice was substantially lower. Migrating to CNS throughout the GALT (167,168), IL-17 expressing Th cells level is essential for neurodegeneration as their interaction with microglia can contribute to the clearance of Aβ and tau aggregations.

Having demonstrated the interaction between the gut microbiota and the inflammatory profile, it has been questioned whether the restoration of gut microbiota by probiotic administration can be an effective treatment strategy for AD. This issue has been studied in different AD experimental models. For example, the administration of SLAB51, a probiotic formulation, to 3xTg-AD mouse model for 4 months reduced

proinflamamtory (IL-1α, IL-1β, IL-2, IL-12, IFN-ɣ and TNF-α) and increased the anti-inflammatory cytokine (IL-4, IL-6….) levels in plasma (169). Furthermore, another study evaluated the effects of Fructooligosaccharide (FOS) on inflammation, Aβ load and behavioral disorders, antioxidative system and energy metabolism in Sprague-Dawley AD models (170). This work found that elevated rates of proinflamamtory cytokines returned to normal rates with FOS therapy promoting the growth of *Bifidobacteria* and *Lactobacilli*, metabolites of which may increase plasma levels of anti-inflammatory cytokines (169).

4.2 Gut and blood brain barrier permeability

Gut microbiota impairments may lead to intestinal inflammation, reducing the expression of tight junction proteins in epithelial colon cells. The loss of gut barrier function causes crossing of the microbial exudates to the circulatory system, which triggers the inflammatory response. Thus, aberrant amounts of plasma proinflamamtory cytokines may enter the brain via the BBB and cause inflammation by altering microglial maturation (171) and astrocyte activation (172).

It has been hypothesized that gut and subsequent BBB leaking could be the underlying mechanism linking obesity and AD. According to this hypothesis, in a study where mice were fed with a diet enriched in unsaturated fatty acids, a profound gut microbiota alteration was observed (24). Moreover the group treated with diet had an increase of gut permeability, presumably due to the decreased expression of both ZO-1 and occludin proteins, corresponding to higher circulating rates of the bacterial LPS and also of inflammatory markers, such as IL-1 and TNF-α. Interestingly, oral antibiotic administration restored gut microbiota and the gut permeability by increasing the expression of both ZO-1 and occludin proteins (24). In line with this idea, Zhang et al. found that patients with neurodegeneration had increased serum LPS and monocyte activation levels compared to controls (173). Since it is recognized that the BBB is disrupted in AD and this can result in anomalous microglial activation and neuroinflammation, the combination of an impaired permeability at intestinal level could also trigger pathogenic signals between the gut microbiota and the CNS. In fact, microbial dysbiosis that alters the permeability of the gut can cause a systemic inflammatory state that can potentiate the usual neuroinflammatory reactions observed in AD (174,175). In this context, Zhao et al. reported increased concentration of the bacterial LPS in the hippocampus and neocortex of AD patient postmortem brains (176).

In addition to the effect on gut permeability, it has been suggested that the intestinal microbiota might also have a direct impact on certain AD biomarkers (175). Indeed, a recent study highlighted a positive association between a pattern of about 50 microbial metabolites and the initiation of AD and cognitive decline (115). Interestingly, among the increased metabolites, there was also TMAO, a microbial metabolite associated with the consumption of animal fats, thus supporting the hypothesis that HFD may be a risk

factor for AD development also by inducing a gut microbial dysbiosis, which in turn exerts a series of proinflammatory, pathogenetic functions.

5. STRATEGIES TO ALTER TMAO LEVELS

The discovery of TMAO as a metabolite produced by gut microbiota and metabolized in the liver that plays a role in systemic inflammation, atherosclerosis, T2DM and vascular dysfunction, has raised the possibility of treating these diseases by targeting gut microbiota and their metabolites.

The use of prebiotics and probiotics could be useful to elicit a favourable impact on gut microbiota composition. Prebiotics include all types of non-digestible foods, such as oligosaccharides, that stimulate the growth of beneficial bacteria (65), while, probiotics include the administration of specific bacterial strains. Those bioactive foods could be useful to decrease bacteria able to transform precursors into TMA and to increase bacteria able to deplete it or bacteria without the genes needed to convert carnitine or choline into TMA. As an example, although it has to be demonstrated in humans, the administration of *Lactobacillus paracasei* in mice expressing human baby microbiota reduced TMA production (177). Other studies have proposed the use of methanogenic bacteria to deplete TMA and TMAO (178,179). A large number of bacteria belonging to the order Methanobacteriales have been found in the human gut. These Archaea use methyl compounds such us TMA and TMAO as substrate to generate methane (52,70).

Instead of changing the gut microbiota, the option of reducing carnitine or choline levels in the diet would not be a possibility because they are important nutrients and low levels of them can lead to organ dysfunction. Indeed, some of the main functions of choline and its derivatives are the production of neurotransmitters (like acetylcholine) and the stability of the cell membrane (as phosphatidylcholine). Furthermore, L-carnitine helps to maintain skeletal and cardiac muscle function and may be useful in the reduction of major adverse cardiovascular events and mortality after acute myocardial infarction (180).

The use of antibiotics as a therapy to eliminate microbiota able to transform dietary precursors (choline, betaine and L-carnitine) into TMA has also been devised. The use of broad spectrum antibiotics such us ciprofloxacin and metronidazole leads to near almost complete suppression of TMAO levels. However, one month after the withdrawal of antibiotics, TMAO levels are detectable again (58). Other studies performed in mice using a mix of vancomycin, neomycin-sulphate, metronidazole and ampicillin, showed an inhibition of dietary choline-enhanced atherosclerosis. Plasma TMAO levels were supressed and macrophage foam cell formation was inhibited (44). Albeit the use of antibiotics is effective in the suppression of microbiota that produces TMA, the chronic use of antibiotics is not viable since it can lead to resistant bacterial strains and repopulation. Moreover, antibiotics do not only kill harmful bacteria, but could affect the beneficial too (58).

Another possibility could be the use of oral non-absorbent binders to remove TMAO or its precursors. For example, oral charcoal adsorbent (AST-120) has been clinically used to remove uremic toxins such as indoxyl sulfate from patients with advanced renal failure. However, a compound that removes specifically TMAO has not yet been discovered (55,181).

Another appealing approach is the inhibition of the enzymes involved in TMA biosynthesis. The gene cluster responsible for the conversion of choline into TMA has been identified. *CutC* and *CutD* are necessary for that transformation; so they could be used like targets as their inhibition eradicates the production of TMA (55). The inhibition of the enzymes able to generate TMAO could be another option. The knockout of *fmo3* normalizes TMAO levels in mice (182). Nevertheless, there could be several problems with this approach as an accumulation of TMA leads to trimethylaminuria, which is characterized by fishy odor (similar to rotten fish) and induce inflammation (55,183,184). Another problem would be that TMAO is not the only substrate for FMO3; morphine, propranolol and tyramine are also metabolized by FMO3 (55).

Several studies have associated the use of some herbal products with lower plasma TMAO levels. For example, the use of *Gynostemma pentaphyllum* (a plant used in China to treat hyperlipidemias and obesity) seems to reduce plasma TMAO levels and increase lecithin levels in rats (94). On the other hand, the use of Gancao (the root of *Glycyrrhiza uralensis*) seems to reduce TMAO levels when Fuzi (the processed lateral root of *Aconitum carmichaelii*) is co-administered (185). Other compounds such as resveratrol could modulate gut composition, decreasing TMA-forming bacteria and increasing the amount of *Lactobacillus* and *Bifidobacterium*. Thus, plasma TMAO levels decrease. Resveratrol did not show beneficial effects when antibiotics were used concomitantly (186). These studies were performed in mice, but similar results could be expected in humans.

The administration of enalapril has recently been linked to lower plasma TMAO levels in rats, probably by increasing urine TMAO excretion. However, the mechanism is uncertain as enalapril did not achieve to decrease indoxyl sulphate levels (187). This study also demonstrated that enalapril does not affect TMA production or gut bacteria composition.

Other proposed therapeutic approaches include the use of some analogues or the inhibition of TMA precursors. In the study performed by Hui et al. (2016) (61), the inhibition of the phospholipase-autotaxin pathway, which generates choline and lysophosphatidic acid from lysophospholipids such as lysophosphatidylcholine, was suggested.

There are other compounds such as meldonium that has been also studied. Meldonium is an aza-analogue of GBB with cardioprotective effects that is used as an anti-ischemic and anti-atherosclerotic drug. Moreover, it seems to reduce plasma TMAO levels in humans by increasing its urinary excretion and reducing its biosynthesis from L-

carnitine (inhibiting the conversion of GBB into L-carnitine) (188). Contrarily, other studies performed in rats suggest that meldonium decreases the excretion and production of TMAO. It increases GBB levels and reduces TMA formation from L-carnitine, but not from choline (189).

One of the most studied strategies is the use of 3,3-dimethyl-1-butanol (DMB) to prevent TMA formation (Table 3). DMB is an analogue of choline that inhibits choline TMA lyase. DMB can be found in some foods like balsamic vinegars, red wines or extra-virgin olive oil and grapeseed oil (190). Although it inhibits the transformation of choline, carnitine and crotonobetaine into TMA in mice and rats, DMB is not able to avoid the complete TMAO synthesis as it cannot inhibit the conversion of GBB to TMA (191). This way, through inhibiting formation of TMA in the gut, TMAO formation in liver is also prevented.

DMB is closely related to inflammation as several studies have showed DMB decreases levels of pro-inflammatory cytokines such us IL-1β, IL-2 IL-6, IL-17 and TNF-α and raises levels of anti-inflammatory cytokines such us IL-10 (45,71,192–196)..

Furthermore, DMB seems to exert an effect on eNOS, reducing superoxide production and increasing NO production, what could block damage caused by cytokines. It also reduces NO scavenging by superoxide (197). Other studies have also showed that DMB increases the phosphorylation of eNOS at Ser1177, what activates eNOS and it may also raise eNOS total expression. Finally, DMB also showed to prevent vascular dysfunction associated to aging or choline-related cardiac dysfunction and fibrosis (197).

Regarding to AD, a recent study (193) showed that DMB treatment led to a significant decrease in the concentration of Aβ1-42, β-secretase, and the β-cleaved carboxy-terminal fragment of APP (βCTF) in the hippocampus of APP/PS1 mice. Therefore, DMB could be considered as a promising therapy for neurodegenerative diseases.

Table 3. Results of DMB in TMAO-induced alterations

Experimental model	Effect on TMAO	Results after treatment with DMB	Reference
Control vs DMB Balb/c mice	Lowers TMAO	↓ IL-1β and IL-6 ↑ VEGF in the ischemic muscle ↑cGMP ↓MDA Better perfusion recovery after experimental PAD	(194)
High choline diet (+ 1.2% choline) C57BL/6 mice with Heart failure with preserved ejection fraction	Lowers TMAO	DMB reverses Choline induced cardiac dysfunction DMB reverses Choline induced cardiac fibrosis DMB reverses Choline induced cardiac inflammation ↓TNF-α, IL-6, and IL-1β	(196)
Control vs DMB C57BL6/J male mice with heart failure	Lowers TMAO	DMB improved cardiac function via TGF-β1/Smad3 signaling pathways DMB attenuated the expression of inflammatory cytokines TNF-α, IL-6, and IL-1β in mice after AB surgery by inhibiting p65 NF-κB signaling pathway	(195)
9 month-old APP/PS1	Lowers TMAO only in APP/PS1not in WT	DMB treatment led to a significant decrease in the concentration of Aβ1-42, β-secretase, and βCTF in the hippocampus of APP/PS1 DMB treatment significantly reduced the concentration of clusterin in plasma ↓TNF-α, IL-2, and IL-17 in hippocampus	(193)
High fructose diet (HFrD) SD rats	Lowers TMAO vs control and HFrD	DMB treatment protected HFrD induced hypertension Prevents ↑ acetate from HFrD ↓ propionate levels ↑ renal mRNA expression of Olfr78 and GPR91	(198)
Myocardial infarction (MI) rats	Lowers TMAO in control and MI rats	Plasma IL-8 levels were restored in MI rats ameliorating MI	(199)
CKD rats	Lowers TMAO in both Control and CKD	Normalized phosphorylation of eNOS and superoxide production in CKD group without effects in sham group Did not alter expression of TNF-α and IL-6 in sham group but reduced the expression of both inflammatory cytokines in CKD group to the same extent as those observed in sham-vehicle group	(200)
LFD vs HFD C57BL/6 mice	Lowers TMAO in both LFD and HFD	Prevented renal interstitial fibrosis induced by HFD ↓ expression of p-SMAD3 in HFD but not in LFD Prevented increases in expression of KIM-1 and plasma levels of cystatin C in mice fed a HFD but had no effects in mice fed a LFD Prevented the ↑ expression of NOX-4, TNF-α and IL-1 β induced by HFD No effect in LFD	(76)
Young and old Fisher Rats	Lowers TMAO in both old and young	↓TNF-α, IL-1β in old group Restored eNOS to normal levels in old group	(71)

Western diet (WD) CD1 mice	Lowers TMAO in both ND and WD	Prevents ↑ TNF-α, ↑IL-1β and ↓ IL-10 caused by WD. No effect in ND. ↓ interstitial fibrosis in the hearts of WD mice. No effect in ND	(45)
Supplementation with TMAO Young and old C57BL/6 N mice	Lowers TMAO in old but not in young	Reversed age-related reductions in EDD with no effects in young mice. Restores endothelial function in old mice by enhancing NO bioavailability. Improves NO-mediated endothelial function with aging by both increasing NO production via eNOS and reducing NO scavenging by superoxide.	(197)
Choline supplementation C57BL/6 mice	Lowers TMAO	Reversed choline related cardiac fibrosis.	(201)
Normal pregnant (NP) vs preclampsia model (RUPP)	Lowers TMAO in both NP and RUPP	Reversed impaired vasodilation and hypertension ↑ IL-10 in both NP and RUPP ↓TNF-α, IL-1β only in RUPP ↓ superoxide production ↑p-eNOS and total eNOS in RUPP	(192)
Middle-aged (18 mo) male C57BL/6 mice	Lowers TMAO	Prevents vascular dysfunction associated to aging	(197)

↑:increase, ↓:decrease. ND: Normal Diet; HFD: High Fat Diet; LFD: Low Fat Diet; NO: Nitric Oxide WD: Western Diet; WT: Wild type.

HYPOTHESIS AND AIMS

HYPOTHESIS AND AIMS

1. JUSTIFICATION OF THE STUDY

Obesity is characterized by an imbalance between caloric consumption and energy expenditure, which promotes adipose tissue expansion. Obesity leads to remodeling of adipose tissue and ultimately to unresolved chronic inflammation. It has become a worldwide problem that can lead to a major number of diseases including insulin resistance, T2DM and even neuropathologies.

On the other hand, AD is the most common form of dementia, which is also closely related to age. It is also one of the main causes of mortality in aging people. Its etiology remains unknown, but it is thought to be a multifactorial disease with aging being the greatest risk factor. There is no actual treatment to cure or even retard the progression of the disease; the only available treatment is to alleviate symptoms. For this reason it is essential to understand the alterations and changes that takes place during aging and find new therapies and strategies to try to reverse the cognitive decline.

In light of the high numbers of overweight/obese and diabetic individuals, there is a clear need to better understand the pathophysiological mechanisms underpinning insulin resistance/obesity and the accompanying impact on cognitive function. Inflammation is suggested to be an important pathophysiological mechanism underlying cognitive impairment and dementia, and has been involved in the neuropathological hallmarks of AD (202). On the other way, it is well accepted that obesity and peripheral insulin resistance are linked to low-grade inflammation in peripheral tissues and the circulation (21,203). This inflammation may eventually spread from peripheral tissue to the brain (204). Thus, whilst several mechanisms are likely to associate peripheral metabolic disturbances and cognitive impairment, it might be hypothesized that systemic and central inflammation may converge into a final common pathway leading to cognitive dysfunction. Moreover, emerging evidence has implicated the gut-brain axis as playing a crucial role in the relationship between insulin resistance/obesity and cognitive dysfunction. The gut microbiome has a considerable impact on brain function (205,206) and is profoundly affected by dietary factors (207,208).
Gut microbiota can synthesize a range of compounds that could mediate local and systemic inflammation. In recent years, TMAO has emerged as a pro-inflammatory molecule closely related to obesity and aging, both of which are nowadays considered main risk factors for AD. In this context, inhibition of TMAO formation may be a promising therapy to prevent neuroinflammation.

2. HYPOTHESIS

The present study analyzes the hypothesis on wherein gut microbiota affect the pathogenesis of AD. Emerging evidences show that there is a relationship between the biology of AD risk factors, such as aging or obesity, and the pathophysiology of AD. Risk factors, can induce microbiota dysbiosis and when the impairments in bacterial

taxa reach to a level that proinflamamtory bacteria abundance becomes higher than anti-inflammatory bacteria, bacterial metabolites such as TMAO trigger gut and BBB leaking, inducing systemic and CSN inflammation and subsequent neurodegeneration. Based in this idea, this work aims to assess if decreasing TMAO levels by DMB may be a promising therapeutic strategy for neurodegenerative diseases.

3. AIMS

The main aim of this study is to elucidate if TMAO could be the linking mechanism between cognitive decline in AD and its risk factors (i.e. insulin resistance and aging), as wells as to determine the efficacy of DMB preventing TMAO adverse outcomes and reversing cognitive deficits.

To achieve the main objectives the specific aims are:

1 DETERMINATION OF THE ROLE OF TMAO IN PERIPHERAL INFLAMMATION AND NEUROINFLAMMATION

In this section we aim to study *in vitro* the effects of TMAO in different cell lines.

1.1 Study the impact of TMAO on adipocytes (3T3-L1 cell line)
1.2 Assessment of the effect of TMAO on peripheral inflammation (macrophages, RAW264.7 cell line)
1.3 Analysis of TMAO effects in BBB integrity (hCMEC cells)
1.4 Evaluation of the potential pro-inflammatory effects of TMAO (using primary neuronal and microglia cultures)

2 STUDY THE BEHAVIORAL AND MOLECULAR INVOLVEMENT OF TMAO IN AGING

The role of TMAO in aging will be studied in SAMP8 model at three ages: 2-months-old SAMP8 (young mice), 6-months-old SAMP8 (adult mice) and 10-month-old SAMP8 (old mice). Moreover, TMAO levels will be measured in CFS samples of human aged subjects.

The specific objectives are to:

2.1 Evaluate the effect of aging in different peripheral outcomes that may impact cognitive function (glucose tolerance, insulin resistance, adiposity…)
2.2 Characterize the behavioral consequences of aging
2.3 Assess BBB integrity
2.4 Study the effects of aging in inflammation
2.5 Analyse age-induced gut dysbiosis
2.6 Study TMAO levels in serum and brain of aged mice
2.7 Determine TMAO levels in CSF samples

3 BEHAVIORAL AND MOLECULAR CONSEQUENCES OF TMAO INHIBITION USING DMB

This section aims to study whether TMAO inhibition, with a treatment with DMB, is able to prevent the age-dependent progression of the pathology in the SAMP8 model specifically.

3.1 Evaluate the effects of DMB on the main hallmarks of peripheral status (insulin resistance, glucose tolerance and adiposity)
3.2 Evaluate the cognitive consequences of TMAO inhibition
3.3 Evaluate the effects of DMB in alleviating inflammation

EXPERIMENTAL DESIGN AND METHODS

EXPERIMENTAL DESIGN AND METHODS

1. IN VITRO ASSAYS

TMAO was dissolved in UHP water to obtain a stock solution of 2 mM. For cell viability, the following final concentrations were tested: 10, 50, 100, 200, 300, 400, 500, 700 and 1000 μM.

1.1 Cell cultures

1.1.1 Adipocytes (cell line 3T3-L1)

3T3-L1 mouse pre-adipocytes (ATCC® CL-173™, Rockville, USA) were cultured at 37ºC, in humidified air with 5% CO_2, in Dubbelco's Modified eagle Medium (DMEM; gibco, Life Technologies, Paisley, UK) supplemented with a solution 1% of Penicillin/Streptomycin (Lonza 17-602E) and 10% of calf bovine serum (CBS).

Cells were grown in tissue culture flask of 75 cm^2 (BD Falcon, Franklin Lakes, NJ, USA). The medium was changed every 2-3 days and when a confluence of 70% was reached, cells were seeded in 6 well cell culture clusters (Costar, Corning, NY, USA). 3T3-L1 cells present a fibroblast like morphology, but in the presence of a hormone cocktail they acquire adipocyte-like phenotype. When a full confluence was reached in the 6 well culture cells clusters, medium culture was removed and the differentiating hormone cocktail was added, consisting of DMEM (Gibco, Life Technologies, Paisley, UK) supplemented with a solution 1% of Penicillin/Streptomycin (Lonza 17-602E), 10% of fetal bovine serum (FBS; gibco Life Technologies, Paisley, UK) 10 μg/mL insulin, 0.5 mM Isobutylmethylxanthine and 1 μM Dexamethasone.

3T3-L1 cells stayed in this medium for 2 days and changed to DMEM (Gibco, Life Technologies, Paisley, UK) supplemented with a solution 100 U/mL of Penicillin/Streptomycin (Lonza 17-602E), 10% of fetal bovine serum (FBS; gibco Life Technologies, Paisley, UK) and 10 μg/mL insulin for 2-4 days to round the adipocytes. Then, adipocytes were maintained in DMEM (Gibco, Life Technologies, Paisley, UK) supplemented with a solution 1% of Penicillin/Streptomycin (Lonza 17-602E) and 10% of fetal bovine serum (FBS; gibco Life Technologies, Paisley, UK) and without hormones until 70% cells exhibited the mature adipocyte phenotype.

1.1.2 Macrophages (cell line RAW 264.7)

Murine RAW 264.7 macrophages (ATCC® TIB-71™) were cultured at 37ºC, in humidified air with 5% CO_2, in DMEM (Gibco, Life Technologies, Paisley, UK) supplemented with a solution 1% of Penicillin/Streptomycin (Lonza 17-602E) and 10% of fetal bovine serum (FBS; gibco Life Technologies, Paisley, UK).

The medium was changed every 2-3 days and cells were split at a confluence of 80%. Cells were grown in tissue culture flask of 75 cm^2 (BD Falcon, Franklin Lakes, NJ, USA). All cells cultivated were below passage 12.

1.1.3 BBB cells (cell line hCMEC/D3)

Human Cardiac Microvascular Endothelial Cells (hCMEC/D3) were cultured at 37ºC, in humidified air with 5% CO_2, in Endothelial Cell Growth Basal Medium (EBM; Lonza, CC-3156) and supplemented with Endothelial Cell Growth Medium SingleQuotsTM Supplements (Lonza, Teknovas, CC-4176) to acquire BBB-like characteristics.

The medium was changed every 2-3 days and cells were split at a confluence of 80%. Cells were grown in tissue culture flask of 75 cm2 (BD Falcon, Franklin Lakes, NJ, USA) previously treated with a mixture of Collagen Type I (Sigma, C3867) and sterile water (1:25). To assure adherence of collagen to flask, they were treated overnight at a temperature of 4-8ºC or 2-3 hours at a temperature of 37ºC. All experiments were performed on cell passage below 8.

1.1.4 Primary neuronal cultures

Primary neuronal cultures were derived from the hippocampus and cortex of embryonic day 16 (E16) mice. Brain tissue was triturated using glass pipettes until neurons were dissociated. Tissue was then placed in a tube with 2 mL of trypsin for 10 minutes at 37ºC. Once the tissue had precipitated, the trypsin was taken out. The tissue was further washed with optimen supplemented with glucose and Penicillin/Streptomycin (Lonza 17-602E) and plated in 6 well plates. After 2 hours, optimum was changed to serum-free neurobasal media with B27 supplement (Invitrogen, Gaithersburg, MD) and 2 mM L-glutamine on poly-L-lysine-treated (0.1 mg/ml; Sigma) 60 mm dishes. Primary neurons were viable for >3–4 weeks under our culturing conditions.

1.1.5 Primary microglial cultures

Primary microglial cultures were obtained from adult mice. Briefly, cortex was dissected out and cut into pieces (200 µm) with a tissue chopper. Tissue was then transferred to a dish with un-supplemented DMEM. Then, tissue underwent enzymatic digestion with papain for 30 minutes at 32ºC with shacking at 200 rpm. Papain was previously prepared dissolving 20 mg of papain in 6 mL of un-supplemented DMEM. Each hemisphere was digested with 6 mL of papain. Once digested, 2 minutes were waited so the tissue settled. Afterwards, tissue underwent mechanical trituration with a pipette while staying in 2 mL of DMEM supplemented with 15% FBS and 1% Penicillin and Streptomycin; the supernatant was recovered. This step was repeated 3 times. Once the supernatant was recovered, it was centrifuged at 397 g for 5 minutes at room temperature and the pellet was resuspended in fresh media (DMEM + 15% FBS + 1% Penicillin/Streptomycin) using filters to filter away long debris. Hoechst dye was used to count cells in the hemacytometer. Finally, 40,000 cells were seeded per well. After 2 hours cells were rinsed twice with un-supplemented DMEM (microglia will remain in the wells) and treatments were added to each well.

1.2 Differentiation assay

Visual differentiation of the 3T3-L1 cells treated with different concentrations of TMAO (50, 200, 400 and 700 µM) was evaluated using the differentiation assay.

3T3-L1 cells were seeded in 6-well plates and once a differentiation of 70% was reached they were treated with TMAO at different concentrations for 24 hours at 37°C in humidified air with 5% CO2. After exposure to treatments, cells were rinsed with PBS solution and left growing undisturbed in fresh 10% FBS supplemented DMEM for 2 days.

Mature differentiated cells were counted 48 hours post-treatment (differentiation) using trypan blue-based specific chambers (Life technologies, C10228) for Countess® automated cell counter (Life technologies).

1.3 Cell metabolic activity assay

MTT assay was used for determining mitochondrial dehydrogenase activity in the living cells. MTT also known as (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium is reduced to a purple formazan by NADH. This formazan is insoluble in water but soluble in organic solvents like DMSO and can be detected at 540 nm using a spectrophotometer.

When cells reached a confluency of 70-80%, the medium was removed and the treatment (200 µL of TMAO at different concentrations) was added to each well, in an ascendant concentration way. After a 24-hour treatment, cell metabolic activity was checked using the MTT study.

0.5 mg/mL MTT solution was added (sigma, M5655) to each well. After 2 hours incubation at 37ºC, medium was removed and the precipitated formazan was dissolved in pure DMSO. Spectrophotometric reading was conducted at 540 nm (Spectra MR, Dynex technologies). Survival was calculated as a percentage relative to the control sample.

1.4 Immunocytochemistry

Microglial primary cells incubated in 4 well plates were fixed with 1% paraformaldehyde (PFA) for 10 minutes; 500 µL of 1% PFA were added directly into the wells. After that, media and PFA were removed and 200 µL of cold methanol (-20ºC) was added for 3 minutes.

Cells were washed 3 times with PBS and primary antibody (Table 4) was added (120 µL/ well) and incubated for 2 hours at room temperature.

Later, cells were washed 3 times with PBS and secondary antibody (Table 4) was incubated in dark for 45 minutes. Finally, cells were washed again 3 times with PBS and cells were flipped and coverslipped with less than 1 drop of Fluorsave Reagent mixed with 0.5 µL of blue Hoechst.

Table 4. List of antibody used for immunocytochemistry

Name	Dilution	Reference
IBA-1	1:1000	Abcam (ab5076)
CD16/CD32	1:100	BD Biosciences (553142)
Alexa fluor 546 donkey anti-goat	1:500	Invitrogen
Alexa fluor 488 donkey anti-rat	1:500	Invitrogen
Alexa fluor 488 donkey anti-goat	1:500	Invitrogen

1.5 Myelin phagocytosis assay

100 µL of rat myelin were mixed with 25 µL of Dil dye (Cat 60010, Lot 981214) and incubated at 37ºC for 5 minutes. Then, 1 mL DPBS/UPH water was added and the mix was sonicated for 15 seconds approximately until homogenization was achieved. After that, myelin was centrifuged at 16000 g for 3 minutes at room temperature. Pellet was resuspended in 100 µL UHP water (0.50 mg/mL of myelin). For the cell treatment, 30 µL of myelin were mixed with 720 µL of DMEM + 15% FBS+ 1% Penicillin/Streptomycin.

Cells were previously seeded in 24 well plates and 60 µL of myelin + medium were added to each well and incubated at 37ºC, in humidified air with 5% CO_2 for 24 hours. Then, primary microglial cells were stained with goat IBA1 and nucleus was stained with blue Hoechst.

1.6 Western blot

hCMEC/D3 cells were rinsed with a PBS solution and collected after a 24-hour treatment. Cells were centrifuged at 2000 rpm for 5 minutes and supernatant was removed. Pellets were stored at -80 ºC.

Cells were homogenized in a lysis buffer and kept cold in ice for 30 min. Then, cells were centrifuged at 13000 rpm for 20 minutes and supernatant was collected.

Equal quantities of protein (50 µg/lane) were separated by electrophoresis on 7.5 % SDS-polyacrylamide gels and consecutively electrophoretically transferred to nitrocellulose blotting membranes (Cat No: 10600048, Amersham). After blocking with Intercept Blocking Buffer (Part No: 927-70001, LI-COR, USA) or 5% milk in TBST for 1 hour at room temperature for binding nonspecific sites, membranes were subjected to immunoblotting with primary antibodies overnight at 4 °C (Table 5).

Table 5. List of antibody used for Western Blot determination

Name	Dilution	Reference
Ocluddin	(1:1000)	40-4700, Thermo Fisher
ZO-1/TJP1	(1:1000)	40-2200, Invitrogen
β-actin	(1:5000)	A1978, Sigma

1.7 RNA extraction and rt-qPCR

Total RNA was extracted using TRI reagent (T9424, Sigma-Aldrich). cDNA was synthesized using a high-capacity cDNA Reverse Transcription kit (4368814, Applied Biosystem). The mRNA expression of GAPDH (Mm99999915_g1), IL-6 (Mm00446190_m1), IL-1β (Mm00434228_m1) and TNF-α (Mm00443258_m1) were quantified by TaqMan-based quantitative real-time PCR. Ct values obtained for each gene were referenced to GAPDH (ΔCt) and converted to a linear form using the $2^{-\Delta\Delta Ct}$ term as a value directly proportional to the copy number of complementary DNA and the initial quantity of mRNA.

2. IN VIVO EXPERIMENTS

2.1 Animals

All the experiments were carried out in a total of 66 mice which were divided in 6 subgroups: SAMR1 2 months-old (n=11), SAMP8 2 month-old (n=8), SAMR1 6 month-old (n=12), SAMP8 6 month-old (n=10), SAMR1 10 month-old (n=17) and SAMP8 10 month-old (n=8). Number of animals was dependant on availability. Mice were housed in a specific pathogen-free environment within a temperature (21 ± 1ºC) and humidity (55 ± 1%) controlled room on a 12 hours light/dark cycle. Both strains were fed a standard mouse diet and water ad libitum in University of Navarra (Pamplona, Navarra, Spain).

Peripheral and behavioral tests took place 16 days prior to the sacrifice following the next diagram (Figure 3). Mice were sacrificed the day after the last trial of MWM following 8 hours of fasting.

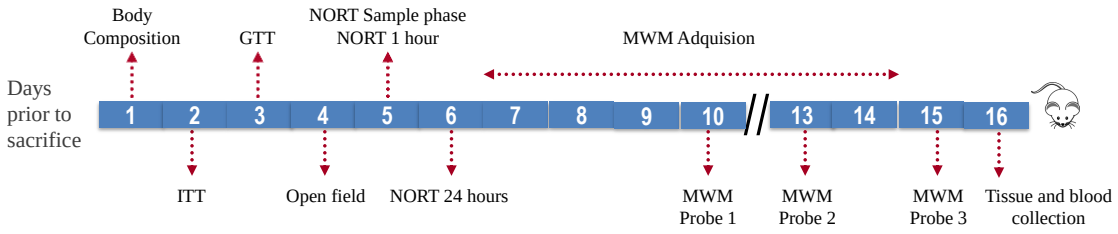


Figure 3. Diagram of peripheral and behavioral test in SAMR1 and SAMP8 mice. Peripheral assays started with body composition test, followed by ITT and GTT. Behavioral tests started 2 weeks prior to the sacrifice with Open field and ended with MWM. ITT: Insulin Tolerance Test, GTT: Glucose Tolerance Test, NORT: Novel Object Recognition Test, MWM: Morris Water Maze.

For DMB treatment, eighteen SAMR1 and eighteen SAMP8 mice aged 6 months were randomly divided into four experimental groups as follows (n=9 per group): SAMR1 control group (R1-C), SAMR1 mice treated with 1% (vol/vol) DMB group (R1-DMB), SAMP8 mice control group (P8-C), SAMP8 mice treated with 1% (vol/vol) DMB group (P8-DMB). DMB (TCIAD1333, VWR, TCI Europe) was given in drinking water for 9 weeks and renewed every week.

Behavioural tests started at the beginning of the 7th week and mice were sacrificed the day after the last trial of MWM following 8 hours of fasting (Figure 4).

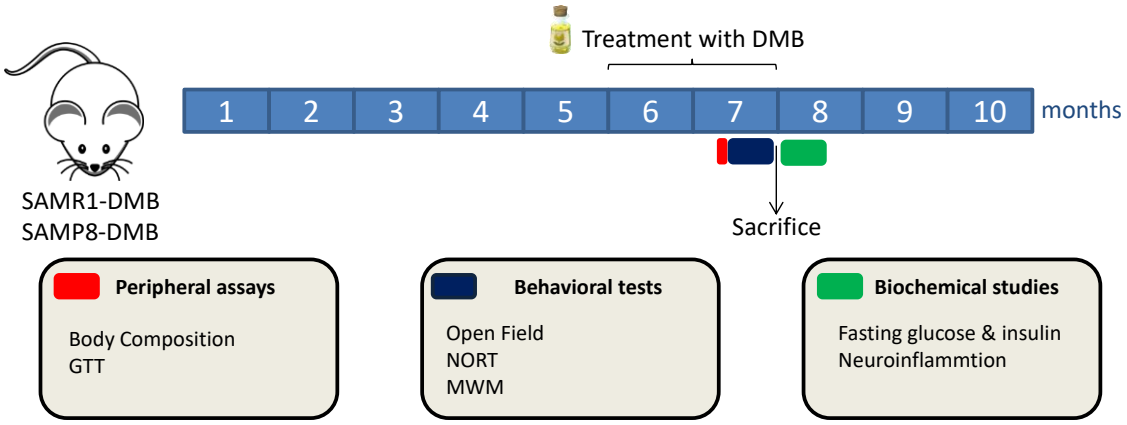


Figure 4. Experimental design of DMB treatment in SAMP8 model. 6 months old SAMP8 and SAMR1 mice were treated with DMB 1% (vol/vol) for 9 weeks. Peripheral assays took place the 7th week and behavioral tests were performed later within the 7th week. GTT: Glucose Tolerance Test, NORT: Novel Object Recognition Test, MWM: Morris Water Maze.

Animal handling and breeding was conducted in accordance with the principles of laboratory animal care as detailed in the European Communities Council Directive (2003/65/EC), Spanish legislation (Real Decreto 1201/2005) and approved by the Ethics Committee of the University of Navarra. In addition, every effort was made to minimize the number of animals used and any possible suffering.

2.2 Body composition assessment

Body composition was determined for all mice of each group using nuclear magnetic resonance (EchoMRI, EchoMedical Systems, Houston, TX, USA) at the end of the study. Scans were performed by placing animals into a transparent plastic cylinder (1.5 mm thick, 4.7 cm diameter), with a smaller plastic cylinder inserted into the big one to limit mouse movement. While in the tube, animals were briefly subjected to a low-intensity (0.05 Tesla) electromagnetic field to measure fat and lean mass.

2.3 Glucose- and insulin-tolerance test

Glucose tolerance test (GTT) was performed after mice underwent a fasting period of 6 hours. Glucose concentrations in blood were measured after the fasting period (0 min), then each mouse received an intraperitoneal injection of 20% glucose (10 ml per kg body weight) and glucose concentrations in blood were measured after 15, 30, 60 and 120 minutes. GTT was conducted at the end of the study, three days previous to behavioral tests. The area under the curve (AUC) of glucose values was assessed for each group from 0 to 120 minutes post glucose injection.

Insulin tolerance test (ITT) was done with mice fed ad libitum. After basal glucose concentrations in blood were measured (0 min), each mouse received an intraperitoneal injection of insulin (0.75 units per kg body weight; Actrapid; Novo Nordisk) and glucose concentrations in blood were measured after 15, 30 and 60 min.

2.4 Behavioral tests

Behavioral experiments were conducted between 09:00-13:00 hours. To perform these tests animals were randomized.

2.4.1 Open field

Open field was used to measure spontaneous locomotor activity and anxiety-like behaviour for 30 minutes in a softly illuminated room. The field was made of black wood (45 cm height x 35cm x 35 cm) and a video-tracking system (Ethovision 11.5, Noldus Information Technology B.V., The Netherlands) was used to analyze distance (cm) and total path velocity (cm/s).

2.4.2 Novel Object Recognition Test (NORT)

NORT was used to test short-term memory, perceptual discrimination and novelty detection.

The open field was composed of a big squared black box divided into four sections (45 cm height x 35cm x 35 cm). The previous day to this experiment, mice were used to the squared sections for 30 minutes. The test consisted in three trials: sample phase, 1 hour trial and 24 hours trial in which exploration time was recorded for 5 minutes using a video-tracking system (Ethovision 11.5, Noldus Information Technology B.V., The Netherlands).

For the sample phase, two identical objects (A and A') were placed in the compartment and mice were allowed to explore the objects for 5 minutes. The next test took place 1h after the sample phase and one of the objects was replaced by a new different one (A and B) and mice were allowed to explore the items for 5 minutes again.

Last trial was performed 24 hours after sample phase; conserving one of the initial items and adding a new one, but in the other position (C and A') trying to avoid position bias.

Results were expressed as percentage of time spent exploring the new object referred to total exploration time (discrimination index). It should be highlighted that the exploration was considered acceptable when the nose of the mouse was oriented within 2 cm of the object.

2.4.3 Morris Water Maze (MWM)

MWM is a hippocampus-dependent task, which was used to evaluate spatial memory and study the working and reference memory functions.

The water maze is composed of a black circular pool (145 cm diameter) filled with warm water (21-22ºC) and virtually divided into four equal quadrants and a hidden platform, which is 1 cm below water. There were four clues situated in the places that divided each quadrant to help mice position and locate the hidden platform. The experiment was carried out in a softly illuminated room and the trials were monitored using a video camera set above the centre of the pool and connected to a video-tracking system (Ethovision 11.5, Noldus Information Technology B.V., The Netherlands)

There was a previous day of habituation with a visible platform in the southwestern quadrant. Six trials were carried out to get mice used to the pool and teach them that they must reach the platform.

To test learning capacity, the following days the platform was hidden 1cm below water and situated in the northeaster quadrant. 4 trials/day were performed for six consecutive days; one from each point. Every trial was finished when the mouse reached the platform (escape latency) or when a maximum of 60 seconds were exceeded. Mice failing to reach the platform were guided onto it and stayed there for 10-15 seconds.

To test memory, probe trials were performed the 4th and the last day (7th). In this probe platform was removed and mice were allowed to swim for 60 seconds. The percentage of time spent in the quadrant where the platform was located previously (northeaster or target quadrant) was recorded. The time spent to reach the place where the platform was located and the number of times mice would cross the platform was also recorded.

2.5 Tissue and blood collection

Mice were killed by decapitation between 09:00–12:00 hours. Brains were removed and dissected on ice to obtain the hippocampus and stored at −80 °C.

For immunohistochemistry assays, left hemispheres from 5 mice per group were fixed by immersion in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 24 hours followed by 20% sucrose solution. Brains were cut into series of 40 μm slides.

Blood was collected, centrifuged at 1250 g (15 min, 4ºC), and serum was frozen at -80ºC.

2.6 Plasma insulin and glucose levels

Fasting glucose was measured with Glucose Assay kit (Abcam ab65333) and fasting serum insulin was determined by Mouse Insulin ELISA (Crystal Chem 90080).

For fasting serum glucose measurement, a colorimetric assay was performed. Briefly, 1 mL of 1 nmol/μL Glucose standard was prepared by diluting 10 μL of the Glucose Standard in 990 μL of Glucose Assay Buffer. Different dilutions of this Glucose Standard were used to build a standard curve.

Firstly, 2.5 μL serum samples were added to a 96-well plate and volume was adjusted to 50 μL with Glucose Assay Buffer. Secondly, 50 μL of Reaction Mix were added into every standard and sample wells. Thirdly, 50 μL of Background Reaction Mix were added into the background control sample wells. Finally, plates were mixed and incubated at 37°C for 30 minutes avoiding light exposure and absorbance was measured at 570 nm.

For plasma insulin determination, low range assay protocol (0.1-6.4 ng/mL) was followed. Briefly, 95 μL of diluent were mixed with 5 μL of serum sample and incubated at 4ºC for 2 hours. Later, plates were washed and 100 μL of the conjugated solution were added and incubated for 30 minutes at room temperature. After that, plates were washed and 100 μL of the substrate solution were added and incubated for 40 minutes. Finally, 100 μL of the stop solution were added and absorbance was measured at 450/630 nm. Fasting serum insulin concentrations were interpolated using the standard curve and mean absorbance values for each sample.

Insulin sensitivity was analysed by assessing the homeostatic model assessment (HOMA) index. HOMA index is expressed as insulin (μU/mL) × glucose (mmol/L) / 22.5.

2.7 Western blotting

Assays were performed in hippocampal tissue as described previously (209). Samples (50 μg of protein) were separated by electrophoresis on a sodium dodecyl sulphate-polyacrylamide gel (7.5%). Membranes were probed overnight at 4 °C with the corresponding primary antibodies (Table 6). Secondary antibodies conjugated to IRDye 800CW or IRDye 680CW (LI-COR Biosciences, Lincoln, NE, USA) were diluted to 1/15,000 in TBS with 5% BSA. Bands were visualized using Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). β-actin was used as internal control. Results were calculated as the percentage of optical density values of the SAMR1 mice.

Table 6. Primary antibodies used for western blot experiments

Protein	Primary antibody (dilution)	Molecular weight	Company
pIR (Y1361)	(1:1000)	90 kDa	Abcam, Cambridge, MA, USA
Total IR	(1:1000)	90 kDa	Cell Signaling Technology, MA, USA
pIRS1 (Ser636/639)	(1:1000)	180 kDa	Cell Signaling Technology, MA, USA
Total IRS1	(1:1000)	180 kDa	Cell Signaling Technology, MA, USA
pAkt (Ser473)	(1:1000)	60 kDa	Cell Signaling Technology, MA, USA
Total Akt	(1:1000)	60 kDa	Cell Signaling Technology, MA, USA
pGSK3β (Ser9)	(1:1000)	46 kDa	Cell Signaling Technology, MA, USA
Total GSK3β	(1:1000)	46 kDa	Cell Signaling Technology, MA, USA
pERK 1/2 (Thr202/Tyr204)	(1:1000)	42/44 kDa	Cell Signaling Technology, MA, USA
Total ERK 1/2	(1:2000)	42/44 kDa	Cell Signaling Technology, MA, USA
p-JNK (Thr183/Tyr185)	(1:500)	46/54 kDa	Cell Signaling Technology, MA, USA
Total JNK	(1:500)	46/54 kDa	Cell Signaling Technology, MA, USA
p-eNOS (Thr495)	(1:1000)	140 kDa	Cell Signaling Technology, MA, USA
Total eNOS	(1:1000)	140 kDa	Cell Signaling Technology, MA, USA
GFAP	(1:1000)	50 kDa	Cell Signaling Technology, MA, USA
Occludin	(1:1000)	59 kDa	Invitrogen–Molecular Probes, OR, USA
Zonulin (ZO-1)	(1:1000)	260 kDa	Invitrogen–Molecular Probes, OR, USA

2.8 Immunofluorescence staining

Serial coronal brain slices (thickness: 40 μm) were cut with a freezing microtome from the frontal cortex till the end of the hippocampus, and were stored in a cryoprotectant solution. Floating tissue sections comprising the hippocampus were processed for immunohistochemistry.

Free-floating brain sections were washed (3 × 10 min) with PBS 0.1 M (pH 7.4) and incubated in blocking solution (PBS containing 0.3% Triton X-100, 0.1% BSA and 2% normal donkey serum) for 2 hours at room temperature. Primary and secondary antibodies were diluted in the blocking solution. Sections were incubated with the

primary antibody overnight at 4°C, washed with PBS and incubated with the secondary antibody for 2 hours at room temperature, protected from light. The primary antibodies used were anti-GFAP (1:250, Cell Signaling Technology, Beverly, MA, USA), anti-fibrin (1: 1000, Dako, Santa Clara, CA, USA), anti-ZO-1 (1: 200, Invitrogen–Molecular Probes, Eugene, OR, USA) and anti-Occludin (1: 200, Invitrogen–Molecular Probes, Eugene, OR, USA). Secondary antibodies used was Alexa Fluor 546 goat anti-mouse (1:200, Invitrogen–Molecular Probes, Eugene, OR, USA) and alexa Fluor 546 goat anti-rabbit (1: 400, Invitrogen–Molecular Probes, Eugene, OR, USA). To visualize brain microvessels, fluorescein-conjugated Lycopersicon esculentum lectin (1: 200, Vector Laboratories, Burlingame, CA, USA) was used and incubated together with the secondary antibody. For better visualization of nuclei, sections were rinsed 10 minutes in the DNA marker TOPRO-3 (Invitrogen–Molecular Probes, Eugene, OR, USA) working concentration 4 μM in PBS, and then washed 2 minutes in PBS before mounting. To ensure comparable immunostaining, sections were processed together under identical conditions. Fluorescence signals were detected with confocal microscope LSM 510 Meta (Carl Zeiss, Oberkochen, Germany).

Images were randomly taken from three non-adjacent tissue sections per specimen (n=4) and analyzed using NIH-developed ImageJ. To quantify capillary leakage, the levels of extravascular fibrin and IgG were measured as previously described (210–213). Briefly, the ImageJ Area tool was used to measure the total area of fibrin- and IgG-positive signal and, when it colocalized with the lectin-positive signal, it was subtracted from the total area of leakage. Using this method, a value representing extravascular levels of each plasma-derived protein is obtained. All images were analyzed by a blinded investigator.

2.9 Real time quantitative PCR assay

Total RNA was obtained from white adipose tissue and brain frontal cortex by homogenizing in TRIzol (Thermo Scientific, Rockford, IL, USA) and following standard methods. RNA quantity and quality were evaluated by a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Rockford, IL, USA). Then, 1 μg of the total RNA of each sample was reverse-transcribed into cDNA using SuperScript III cDNA Synthesis Kit (Thermo Scientific, Rockford, IL, USA). Real-time PCRs were performed using the TaqMan™ Universal Master Mix (Thermo Fisher scientific, MA, USA) in an CFX384 Touch real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). As housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen. The specific primers used for cDNA amplification were interleukin 1β (IL-1β) (Mm00434228_m1), interleukin 6 (IL-6) (Mm00446190_m1) and tumour necrosis factor-alpha (TNF-α) (Mm00443258_m1). Samples were analysed by a double delta CT (ΔΔCT) method. Relative transcription levels ($2^{-\Delta\Delta Ct}$) were expressed as a mean ± standard error of the mean.

2.10 Fecal sample collection, bioinformatics and metagenomic data

Fecal samples were collected using OMNIgene.GUT kits from DNA Genotek (Ottawa, ON, Canada), that is able to stabilize gut microbiome composition and can be kept at

room temperature. CIMA LAB Diagnostics (University of Navarra, Pamplona, Spain) was responsible of bacterial DNA sequencing. In order to characterize the phylogeny and taxonomy of the microbial samples the bacterial 16S RNA gene was sequenced. This gene has approximately 1500 base pairs and contains 9 variable regions in between the conserved regions. For the 16S RNA gene analysis, the V3 and V4 hypervariable regions of the gene were amplified followed by the sequencing, which allowed the assignation up to the species level.

The analyses were conducted using the the Illumina MiSeq equipment, following this protocol: the V3 and V4 regions of the 16S gene are amplified in two PCR reactions that are carried out in a thermocycler, creating an amplicon of approximately 460 base pairs. This requires the use of the 16S-F and 16S-R specific primers (16S Forward Primer =5 0 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; 16S Reverse Primer = 5 0 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCT AATCC). The protocol for the first PCR reaction was the following: 95 °C for 3 minutes, and 25 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds, and finally, 72 °C for 5 minutes, to later keep refrigerated at 4 °C. After the cleansing process, 5µl were extracted from the first PCR reaction sample to use for the second PCR reaction. For the second PCR reaction the employed protocol was 95 °C for 3 minutes, and 8 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds, and finally, 72 °C for 5 minutes, to later keep refrigerated at 4 °C. After each PCR reaction, a cleansing process was carried out to clear the sample from primers. Then, in order to sequence and quantify the samples, they were loaded into the MiSeq equipment.

A code-based approach (barcoding) was used for the complete analysis of the gut microbiome using the OTUs grouping methods. OTU is defined as organisms grouped by similarities in their DNA sequence, with a sequence similarity threshold of at least 75% to 80%. The taxonomy was assigned using BLAST and HITdb, and the sequences were filtered following the OTU LotuS quality criteria (version 1.58). The abundance matrices were filtered and then normalized at each level of classification: OTU, species, genus, family, order, class, and phylum.

In order to perform the comparative analyses to evaluate the differences between gut microbiota composition the MicrobiomeAnalyst tool (https://www.microbiomeanalyst.ca/) was used. For these analyses, the raw count of microorganisms was used. The DESeq2 (RNA-seq methods) analysis was used for finding significant differences in abundance of species and genera, using the trimmed mean of M-values (TMM) normalization and assigning the SILVA taxonomy labels. DESeq2 is a robust method that shows low false positive rates and according to recent guidelines, it has the highest power to compare groups, especially for less than 20 samples per group (214).

2.11 TMAO measurement

Serum TMAO levels were quantified using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) with isotopic marking, using deuterated TMAO standard as described previously (215). Briefly, 20 µL of serum were added to a 1.5 ml Axygen tube containing 80 µL of internal standard mixture of 0.5 µM d9-TMAO in acetonitrile/methanol/formic acid (75/25/0.2 v/v). Once mixed, the sample was vortexed for 30 seconds to precipitate proteins following a centrifugation at 12000 rpm at 4 °C for 15 minutes to recover the supernatant. To elaborate the standard curve, nine samples of known concentrations of TMAO ranging from 0.15 µM to 60 µM were processed using the same procedure reaching a coefficient of determination (R2) above 0.999. Finally, 85 µL of the supernatant were diluted with 20 µL of MilliQ water and once mixed, it was analysed by injection onto a pre-column (X-Bridge BEH Amide 2,5µm, VanGuard Precolumn 2,1 x 5mm, Waters) following a column (X-Bridge BEH Amide 2,5 µm 2,1 x 50mm, Waters) at a flow rate of 0.4 mL/min using UPLC-MS/MS (Waters).

A discontinuous gradient was generated to resolve analytes using phase A (Amonium Acetate 10mmM in MilliQ Water) and phase B (Amonium Acetate 10mmM in acetonitrile/MilliQ water 90/10 v/v) as follows:

Time (min)	Phase A	Phase B
0.0	0%	100%
2.0	50%	50%
3.0	50%	50%
4.0	100%	0%
4.5	100%	0%
5.0	0%	100%
8.0	0%	100%

Analytes were monitored using electrospray ionization (ESI) in positive-ion mode [M-H]+ with multiple reaction monitoring (MRM) of precursors and characteristic production transitions of TMAO at m/z 76→58, d9-TMAO at m/z 85→66, respectively.

For the measurement of TMAO in brain tissue samples approximately 40 mg of parietal cortex were added to a 1.5 ml Axygen tube containing 120 µL of internal standard mixture of 0.5 µM d9-TMAO in MilliQ water. Samples were sonicated in ice with short pulses to homogenize. Supernatant was recovered and 50 µL were mixed with 100 µL of acetonitrile with 1% formic acid. After briefly mixing, the mixture was vortexed for 30 seconds to precipitate proteins following a centrifugation at 12000 rpm at 4 °C for 15 minutes to recover the supernatant. Finally, the supernatant was filtrated using OSTRO plates to get rid of phospholipids and proteins and injected in the column as previously described. To elaborate the standard curve, nine samples of known concentrations of

TMAO ranging from 0.04 μM to 4 μM were processed using the same procedure reaching a coefficient of determination (R2) above 0.999.

3. HUMAN CEREBROSPINAL FLUID SAMPLES

3.1. Sample description

CSF samples were collected in a study conducted by the Karolinska University Hospital (Sweden). 22 patients with subjective cognitive impairment were grouped as controls group because they didn't present complaints on objective cognitive tasks. CSF samples were obtained by lumbar puncture from L3/L4 o L4/L5 interspaces in the mornings. After disposal of the first milliliter, the following 10 mL were collected in polypropylene tubes. No sample containing more than 500 erythrocytes/μL of CSF was used. Samples were gently mixed to avoid gradient effects and centrifuged at 2000 x g at 4ºC for 10 minutes to eliminate cells an insoluble material. Supernatants were aliquoted, immediately frozen and stored at -80ºC.

3.2. TMAO measurement

CSF TMAO levels were quantified using UPLC-MS/MS as described in section 2.11 of Experimental design and Methods.

4. STATISTICAL ANALYSIS

Data analyses were carried out by Stata v.14 for Windows and GraphPad Prism 6. Normality was checked by Saphiro-Wilk's test ($p > 0.05$).

For *in vitro* studies using TMAO, data was exhibited using graph bar showing the mean and the SEM. Student's t-test was used in comparisons between controls and 200 μM TMAO treated samples. One-way ANOVA was used for comparisons between controls and different concentrations of TMAO.

Subsequently, for *in vivo* experiments of the study between TMAO and aging, two-way ANOVA analysis (strain x age) was used. Data was represented by graph bars showing the mean and the SEM. Statistical analysis of MWM acquisition, as well as, ITT and GTT tests was performed by repeated measures ANOVA.

For *in vivo* experiments of the study between TMAO and DMB, two-way ANOVA analysis (strain x treatment) was used. Data was represented by graph bars depicting the mean and the SEM.

Finally, correlation test for CSF human samples was carried out by Spearman test as they showed non-parametrical distribution.

RESULTS

RESULTS

1. ROLE OF TMAO IN PERIPHERAL INFLAMMATION AND NEUROINFLAMMATION: *IN VITRO* STUDIES

1.1 TMAO favoured differentiation of adipocytes from preadipocytes (3T3-L1)

MTT assay showed that increasing TMAO concentrations did not affect negatively cell viability in adipocytes until it reached the concentration of 1000 μM (Figure 5A) (One way ANOVA, $F=4.532$, $p<0.001$). Using the range of concentration that does not induce cell death, TMAO was associated to a major differentiation rate of mature adipocytes from preadipocytes. As shown in Figure 5B, the percentage of differentiated mature adipocytes augmented above 35% when TMAO 200 μM (135.8 ± 4.946) or 400 μM (136.9 ± 6.965) was added to medium (One way ANOVA, $F=5.417$, $p<0.01$). The addition of TMAO 50 μM showed a tendency towards a major differentiation rate but this tendency was not significant.

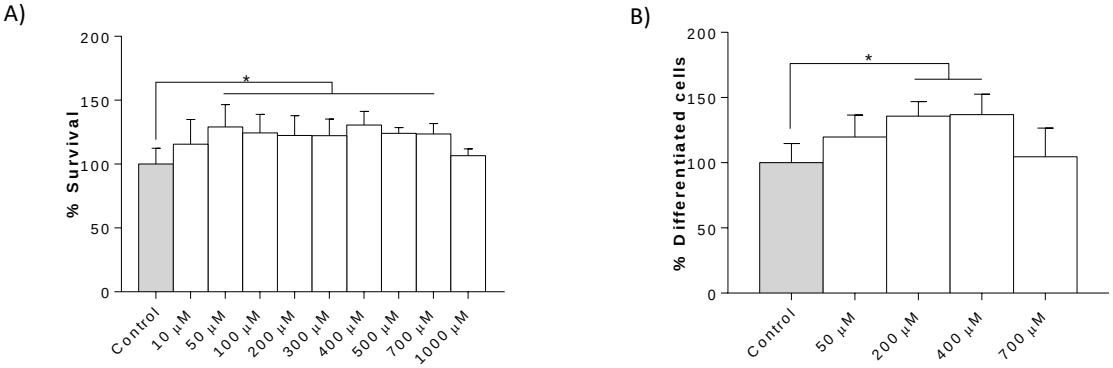


Figure 5. Cell viability and differentiation assay of preadipocytes (3T3-L1) into mature adipocytes. A) Effect of different concentrations of TMAO on cell viability of 3T3-L1 cells. Cells were treated with TMAO for 24 hours. Results are presented as % of survival and expressed as mean ± SEM ($n \geq 8$). One way ANOVA *$p<0.01$ for 100, 200, 300, 500 and 700 μM vs control; *$p<0.001$ for 50 and 400 μM vs control. B) Effect of different concentrations of TMAO on adipocyte differentiation in 3T3-L1 preadipocytes. Cells were treated with TMAO for 24 hours. Results are presented as % of differentiated cells and expressed as mean ± SEM. Five independent experiments were performed. One-way ANOVA, *$p<0.01$.

1.2 TMAO increased expression of pro-inflammatory cytokines in macrophages (RAW 264.7)

Given the demonstrated relationship between TMAO and inflammation, we studied the expression of pro-inflammatory markers such us IL-6, IL-1β and TNF-β in macrophages as they have been shown to infiltrate the adipose tissue and cause inflammation. Once again MTT assay was performed in order to determine the range of concentrations that does not lead to cell death, finding that only 1000 μM decreased cell viability (Figure 6A) (One way ANOVA, $F=4.410$, $p<0.0001$). Interestingly, expression of pro-inflammatory cytokines IL-1β (Figure 6B) (One way ANOVA, $F=6.608$, $p<0.05$), IL-6 (Figure 6C) (One way ANOVA, $F=16.35$, $p<0.001$) and TNF-α (Figure

6D) (One way ANOVA, F=9.207, p<0.01) was found to be augmented after treatment with TMAO 200 µM and 400 µM.

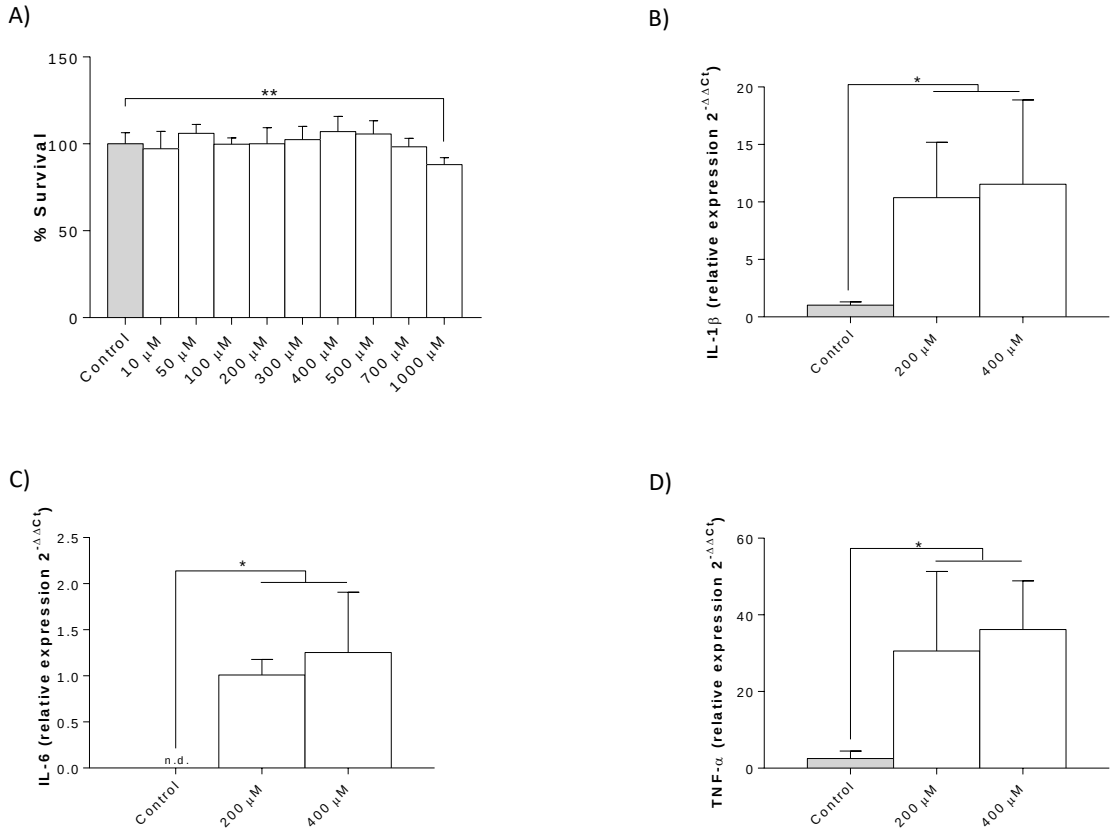


Figure 6. Effects of TMAO on cell viability and pro-inflammatory cytokine release in macrophages. A) Effect of different concentrations of TMAO on cell viability of macrophages RAW 264.7. Cells were treated with TMAO for 24 hours. Results are presented as % of survival and expressed as mean ± SEM (n ≥ 8). One way ANOVA **p<0.01. Effect of different concentrations of TMAO on B) IL-1β, C) IL-6 and D) TNF-α cytokine expression. Cells were treated with TMAO for 24 hours. Results are presented as fold change of protein expression and expressed as mean ± SEM (n ≥ 4). One-way ANOVA, *p<0.01 for IL-1β and TNF-α; *p<0.001 for IL-6.

1.3 TMAO decreased expression of tight junction proteins in hCMEC cells

As depicted in Figure 7A, only the treatment with 1000 µM of TMAO was able to decrease hCMEC cell viability (One way ANOVA, F=9.949, p<0.0001). The treatment with 200 µM of TMAO induced a significant decrease in the expression of occludin (Figure 7B) (Student's t-test, p<0.05) and a strong tendency towards the decrease of ZO-1 (Figure 7C) (Student's t-test, p=0.06).

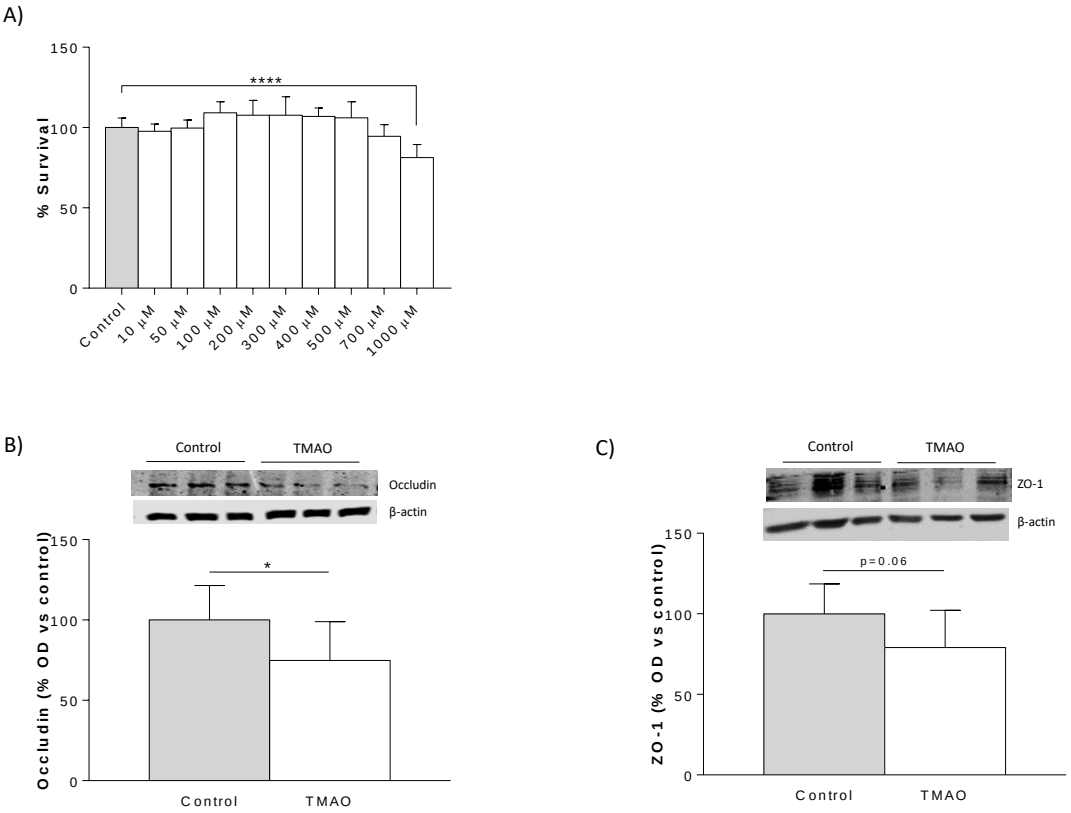


Figure 7. Effect of TMAO on cell viability and tight junction proteins expression in hCMEC/D3 cells. A) Effect of different concentrations of TMAO on cell viability of BBB cells (hCMECs). Cells were treated with TMAO for 24 hours. Results are presented as % of survival and expressed as mean ± SEM ($n \geq 8$). One way ANOVA ****$p<0.0001$. Effect of 200 µM TMAO treatment on B) occludin and C) zonulin expression in hCMEC/D3 cells. Cells were treated with 200 µM of TMAO for 24 hours. Panels show percentage of optical density (OD) values of control and representative pictures of the blotting. β-actin was used as internal loading control. *$p<0.05$ Student's t-test.

1.4 TMAO increased expression of pro-inflammatory cytokines in primary neuronal cultures

In parallel with the observations in other cell types, only 1000 µM of TMAO decreased primary neuronal cell cultures viability (Figure 8A) (One way ANOVA, $F=3.330$, $p<0.01$). Moreover, TMAO treatment induced a significant raise in the expression of IL-1β (Figure 8B) (One way ANOVA, $F=10.89$, $p<0.01$) and IL-6 (Figure 8C) (One way ANOVA, $F=6.379$, $p<0.05$) in primary neuronal cultures. Unexpectedly, no significant changes were observed in TNF-α expression (Figure 8D) (One way ANOVA, $F=3.002$, $p=0.10$).

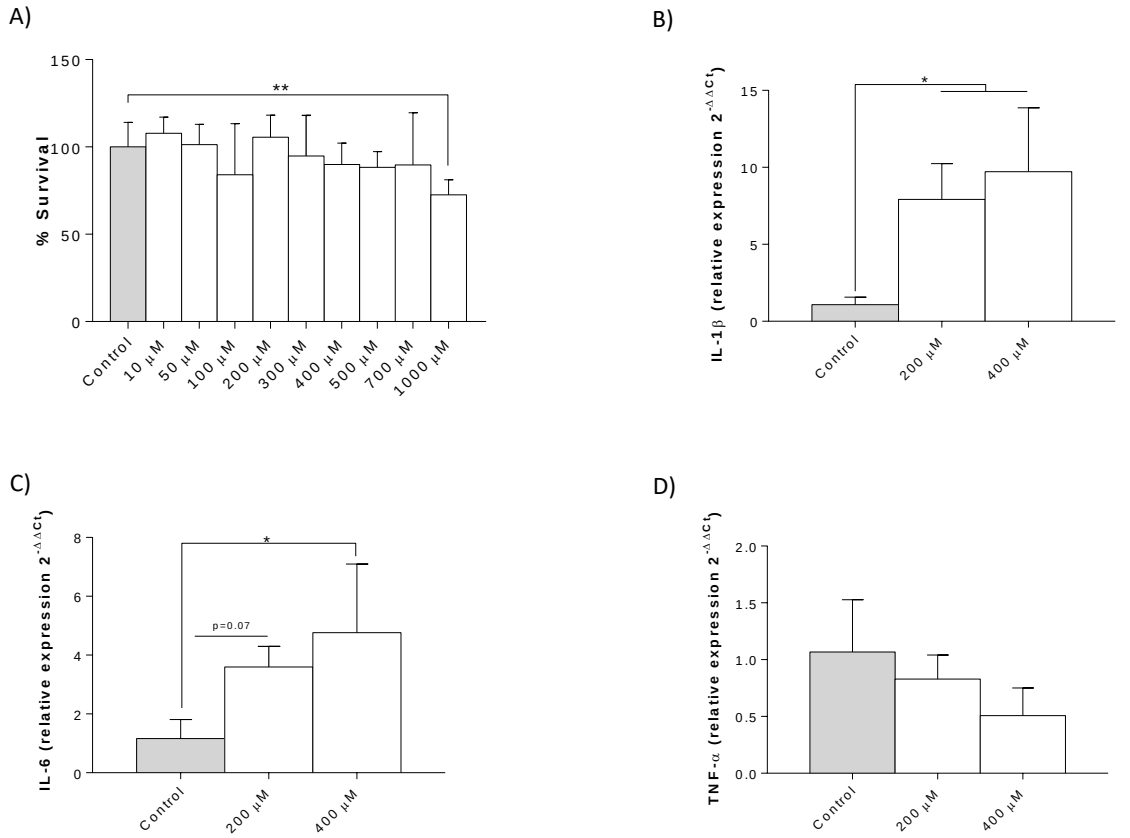


Figure 8. Effects of TMAO on cell viability and pro-inflammatory cytokine release in primary neuronal cultures. A) Effect of different concentrations of TMAO on cell viability of primary neuronal cell cultures. Cells were treated with TMAO for 24 hours. Results are presented as % of survival and expressed as mean ± SEM (n ≥ 8). One way ANOVA **p<0.01. Effect of different concentrations of TMAO on B) IL-1β, C) IL-6 and D) TNF-α cytokine expression. Cells were treated with 200 or 400 µM of TMAO for 24 hours. Results are presented as fold change of protein expression and expressed as mean ± SEM (n = 4). One-way ANOVA, *p<0.05 for IL-1β 200 µM and IL-6; *p<0.01 for IL-1β 400 µM.

1.5 TMAO induced a pro-inflammatory state primary microglia cultures and promotes phagocytosis

TMAO treatment induced an increase in the pro-inflammatory marker CD16/CD32 at all concentrations studied, suggesting a microglial activation towards a pro-inflammatory phenotype (Figure 9A and B) (One way ANOVA, $F=2.874$, $p<0.05$). Moreover, TMAO significantly raised myelin phagocytosis in primary microglial cell cultures (Figure 10A and B) (One way ANOVA, $F=11.14$, $p<0.0001$).

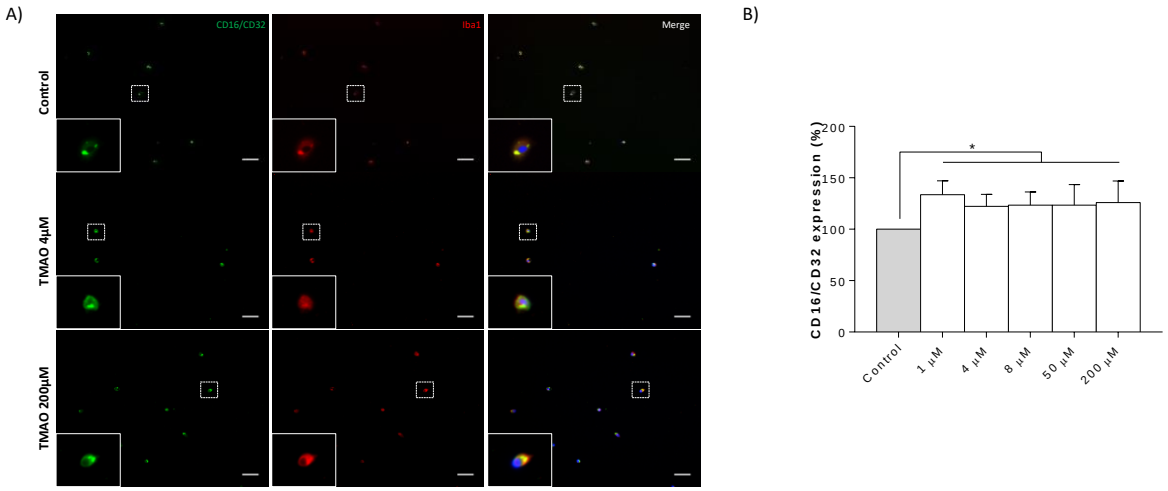


Figure 9. Effect of TMAO on pro-inflammatory activation in primary microglial cultures. A) Immunohistochemical analysis of the effect of TMAO on the pro-inflammatory marker CD16/CD32 expression in primary microglia cultures and B) quantification of the fluorescence intensity. Cells were treated with TMAO for 24 hours. Results are presented as % of normalized CD16/CD32 expression and expressed as mean ± SEM (n ≥ 4). One way ANOVA *p<0.05. Scale bar: 100 µm.

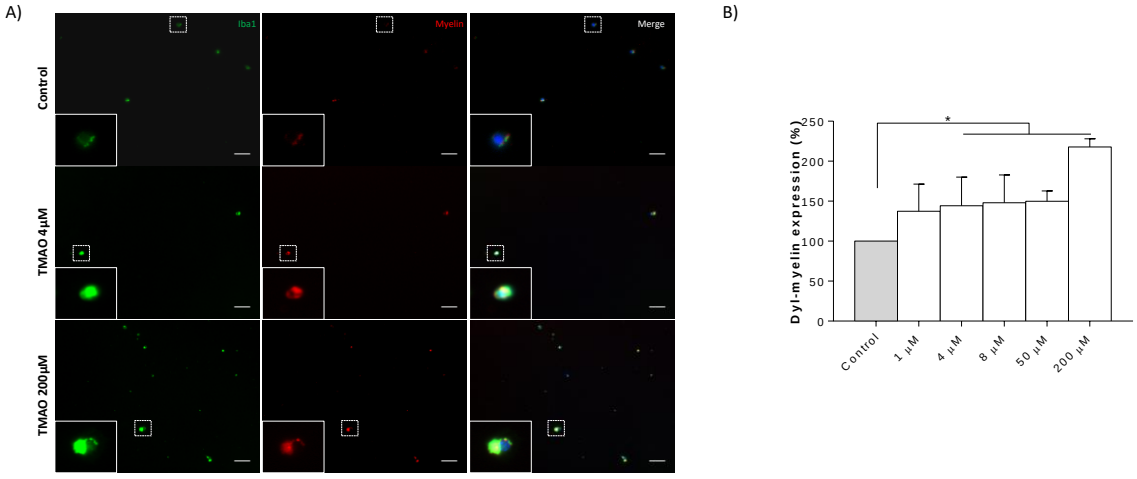


Figure 10. Effect of TMAO on phagocytosis in primary microglial cultures. A) Effect of TMAO on myelin phagocytosis and B) its quantification in primary microglial cultures. Cells were treated with TMAO for 24 hours. Results are presented as % of normalized Dil-myelin expression and expressed as mean ± SEM (n ≥ 4). One way ANOVA *p<0.01 for 4, 8 and 50 µM vs control; *p<0.0001 for 200 µM. Scale bar: 100 µm.

2. ROLE OF TMAO IN THE BEHAVIORAL AND MOLECULAR CONSEQUENCES OF AGING

2.1 Aging alters peripheral insulin sensitivity and glucose homeostasis in SAMP8 mice but not brain insulin signaling

Peripheral insulin sensitivity and glucose homeostasis were assessed by ITT and GTT tests. SAMP8 mice showed enhanced insulin resistance (repeated measures ANOVA, main effect of strain, $F_{5,15}$=5.536; p<0.01) (Figure 11A and B) and glucose intolerance (repeated measures ANOVA, main effect of strain, $F_{5,20}$= 2.263; p<0.05) when compared to SAMR1 mice (Figure 11C and D). Moreover, blood glucose levels increased with age in both strains (two-way ANOVA, main effect of age, $F_{2,58}$=26.21; p<0.0001) but in all time points SAMP8 mice showed elevated glucose concentrations (two-way ANOVA, main effect of age, $F_{1,58}$=6.319; p<0.01) compared to SAMR1 mice (Figure 11E). Surprisingly, concerning insulin and HOMA index only a main effect of age was observed (Two-way ANOVA, $F_{2,58}$=26.21; p<0.0001 and Two-way ANOVA, $F_{2,52}$=0.1630; *p*<0.001, respectively), but although a strong tendency can be observed (especially in the HOMA index), no effect of strain was found, probably due to the high variability of the data (Figure 11F and G).

The observed peripheral metabolism alteration was not due to an increase in SAMP8 mice size or body weight. Indeed, SAMP8 body weight was found to be significantly lower when compared to same age SAMR1 littermates (two-way ANOVA, main effect of strain, $F_{1,61}$=152.4; p<0.0001), although body weight is progressively increased in both strains with the age (two-way ANOVA, main effect of age, $F_{2,61}$=57.66; p<0.0001) (Figure 12A). In order to discover if differences in body weight are due to alterations in body composition, fat and lean mass content were measured in mice. No significant differences were observed between groups in lean (two-way ANOVA, $F_{2,61}$=2.174; p=0.1225) neither in fat mass (two-way ANOVA, $F_{2,61}$=2.837; p=0.0763) (Figure 12B).

In order to study the possible insulin signaling alterations in the brain, expression of several components of the insulin signaling were analysed. Only 10 months SAMP8 mice group showed significant changes in pIRS (Tukey's p<0.05 vs rest of the groups) (Figure 13B) and pAkt levels (Tukey's p<0.05 vs rest of the groups) (Figure 13C). However, no changes were found in the rest of the protein studied (pIR, Figure 13A; pGSK3β, Figure 13D; pERK1, Figure 13E; pERK2, Figure 13F), suggesting that there is not brain insulin resistance. Consistent with a post-transcriptional regulation of these enzymes, total protein levels, normalized using actin, remained unaltered.

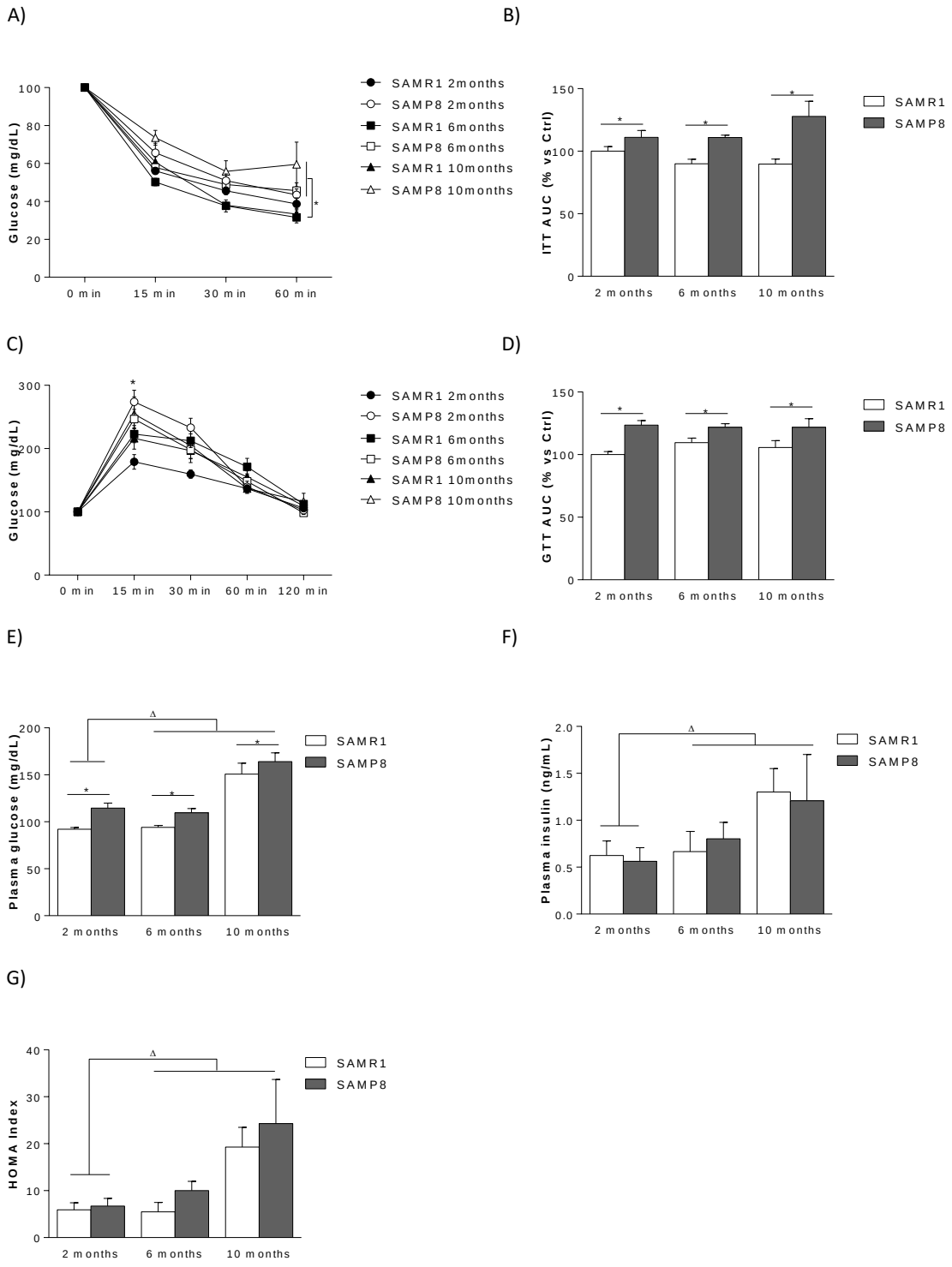


Figure 11. Effect of aging on peripheral insulin sensitivity and glucose homeostasis. The following metabolic parameters were analysed in all six groups. A) Insulin tolerance test (ITT). B) ITT expressed as area under curve (AUC). C) glucose tolerance tests (GTT). D) GTT expressed as area under curve. E) blood glucose levels F) blood insulin levels G) homeostatic model assessment indices of insulin resistance (HOMA). Data are presented as mean ± SEM. *Main effect of strain, two way ANOVA; $^{\Delta}$ Main effect of age, two way ANOVA.

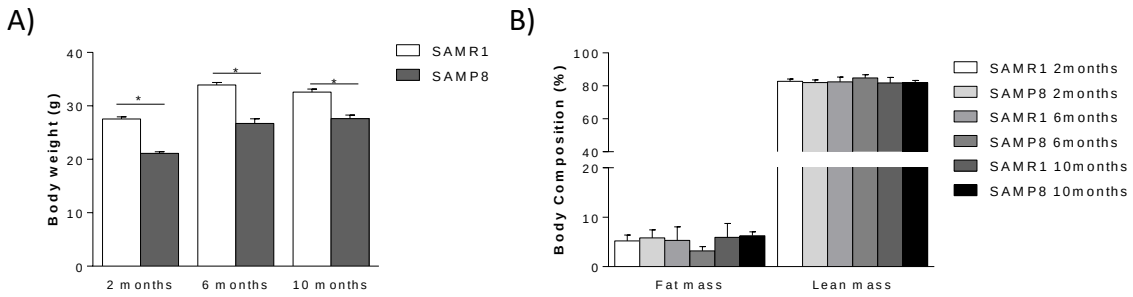


Figure 12. Effect of aging on body composition. In panel A) body weight. In panel B) mice body composition measurement with lean and fat mass assessment respectively. Data are presented as mean ± SEM. *Main effect of strain, two way ANOVA.

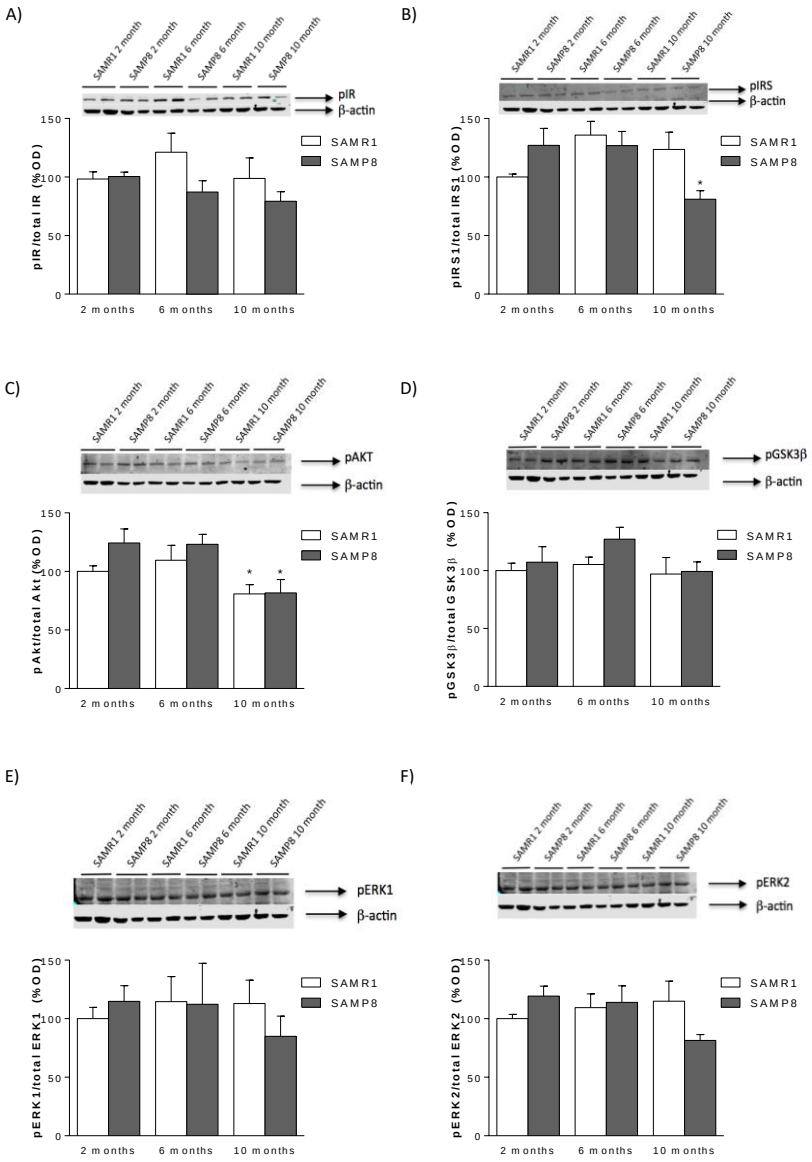


Figure 13. Effect of aging on insulin signaling in the central nervous system. Panel A) pIR expression levels. Panel B) pIRS1 expression levels. Panel C) pAKT expression levels. Panel D) pGSK3β expression levels. Panel E) pERK1 expression levels. Panel F) pERK2 expression levels. Figures show percentage of optical density (O.D.) values of control 2 months SAMR1 mice and representative picture of the blotting. No differences were found in the non-phosphorylated (total) levels of the enzymes. pIR: phosphorylated insulin receptor. *Tukey's multiple comparison test, $p<0.05$.

2.2 Aging induces cognitive deficiencies in SAMP8 mice

As it was expected, age dependent locomotor activity decrease was found (two-way ANOVA, main effect of age, $F_{2,58}$=15.45; p<0.0001) (Figure 14A). Interestingly, there were no differences between strains, indicating that behavioural performance differences between SAMP8 and SAMR1 are not due to locomotor activity alterations.

As shown in figure 14B, during NORT, a main effect of the strain was found (two way ANOVA, main effect of strain, $F_{5,163}$=2.635; p<0.05). Further analysis showed that SAMP8 10 month mice displayed cognitive deficits in the NORT, as shown by a significantly decreased discrimination index in the 1 and 24 hour task (Tukey's p< 0.05 vs rest of the groups). Cognitive impairment was also observed in SAMP8 6 months mice in the 24 hour NORT task (Tukey's p<0.05).

In the acquisition phase of Morris water maze, distance swam to reach the platform improved significantly over trials in SAMR1 group (repeated measures ANOVA, $F_{2,14}$=5.596; p<0.05) but not in SAMP8 group (repeated measures ANOVA, p>0.05). Moreover, significant effect of strain was found (repeated measures ANOVA, main effect of strain, $F_{5,35}$=52.40; p<0.0001) as SAMP8 mice in all ages showed higher scape latency compared to SAMR1 mice indicating a cognitive impairment (Figure 14C). In the retention phase, 6 and 10 months SAMP8 mice showed a statistically significant decrease in time swam in the quadrant were the platform used to be located, indicative of a memory deficit (Tukey's p<0.05 in all cases) (Figure 14D).

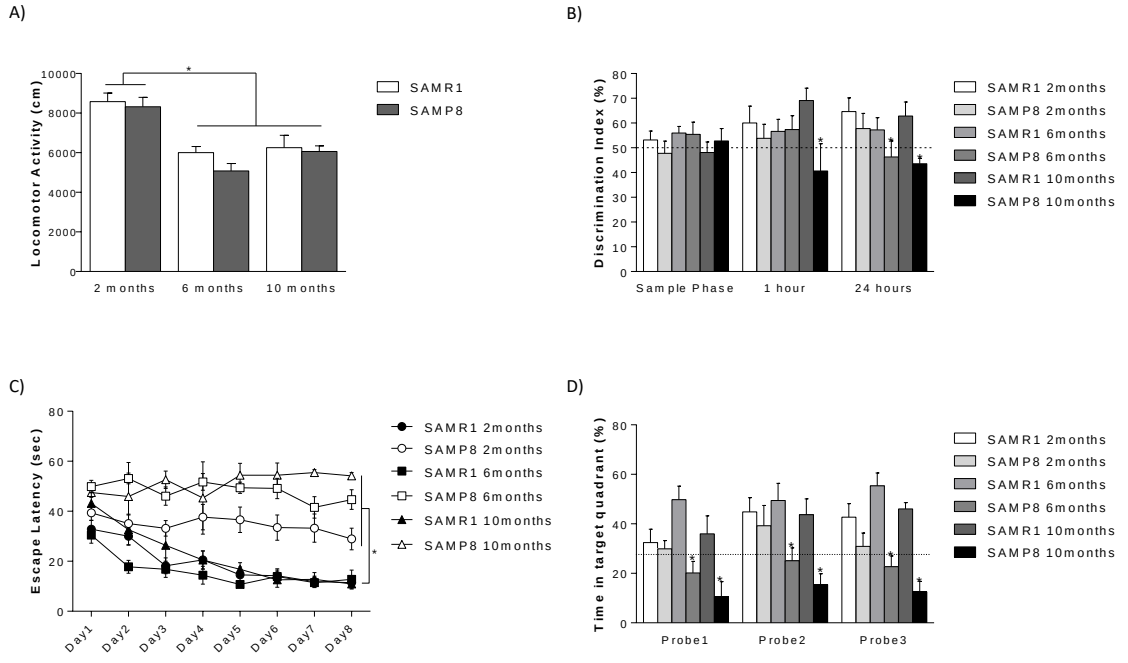


Figure 14. Effect of aging on cognitive performance. In panel A) locomotor activity. In panel B) cognitive performance in novel object recognition test (NORT). Data shows discrimination index (time exploring the new object / total exploration time × 100) in the novel object recognition test. In panel C) and D) cognitive performance assessed by Morris water maze (MWM) acquisition phase and retention phase respectively. Data are presented as mean ± SEM. *Tukey's multiple comparison test, p<0.05.

2.3 Aging disrupts blood brain barrier integrity in SAMP8 mice

BBB integrity was evaluated by immunofluorescence staining of fibrin and IgG in the left hemispheres of SAMR1 and SAMP8 groups. Fibrin (two way ANOVA, main effect of strain, $F_{1,18}$=5.730; p<0.05) (Figure 15) and IgG (two way ANOVA, main effect of strain, $F_{1,12}$=4.641; p<0.05) (Figure 16) extravasation was increased in SAMP8 6 and 10 months aging groups compared to SAMR1 mice .

Moreover, protein levels of two of the main components of the tight junctions (occludin and ZO-1) appeared to be significantly decreased in SAMP8 mice compared to SAMR1 mice (for occludin, two way ANOVA, main effect of strain, $F_{1,30}$=11.31; p<0.01; for ZO-1, two way ANOVA, main effect of strain, $F_{1,30}$=20.46; p<0.0001) (Figure 17).

2.4 Aging promotes peripheral and central inflammation

Our data showed an increase in pro-inflammatory cytokines (TNF-α and IL-6) not only in the periphery (white adipose tissue) (for TNF-α, two way ANOVA, main effect of strain, $F_{1,18}$=6.534; p<0.05; for IL-6, two way ANOVA, main effect of strain, $F_{1,18}$=6.639; p<0.05) (Figure 18A and B) but also in the central nervous system (Frontal cortex, Figure 18C and D) of SAMP8 mice (for TNFα, two way ANOVA, main effect of strain, $F_{1,18}$=4.570; p<0.05 and main effect of age, $F_{1,18}$=4.708; p<0.05; for IL-6, two way ANOVA, main effect of strain, $F_{1,18}$=8.367; p<0.01). Neuroinflammation was further confirmed by p-JNK and GFAP (an astrocyte activation marker) protein levels. As depicted in Figure 19A, a significant increase in p-JNK levels in SAMP8 mice in all ages were observed (two-way ANOVA, main effect of strain, $F_{1,42}$= 27.82; p<0.0001). In addition, an age and strain dependent significant increase in GFAP levels were observed in SAMP8 mice compared to SAMR1 group (two-way ANOVA, main effect of age, $F_{2,41}$=14.05; p< 0.0001; two-way ANOVA, main effect of strain, $F_{1,41}$=6.327; p< 0.05) (Figure 19B). The increase in GFAP was also observed by immunohistochemistry analysis (Figure 19C).

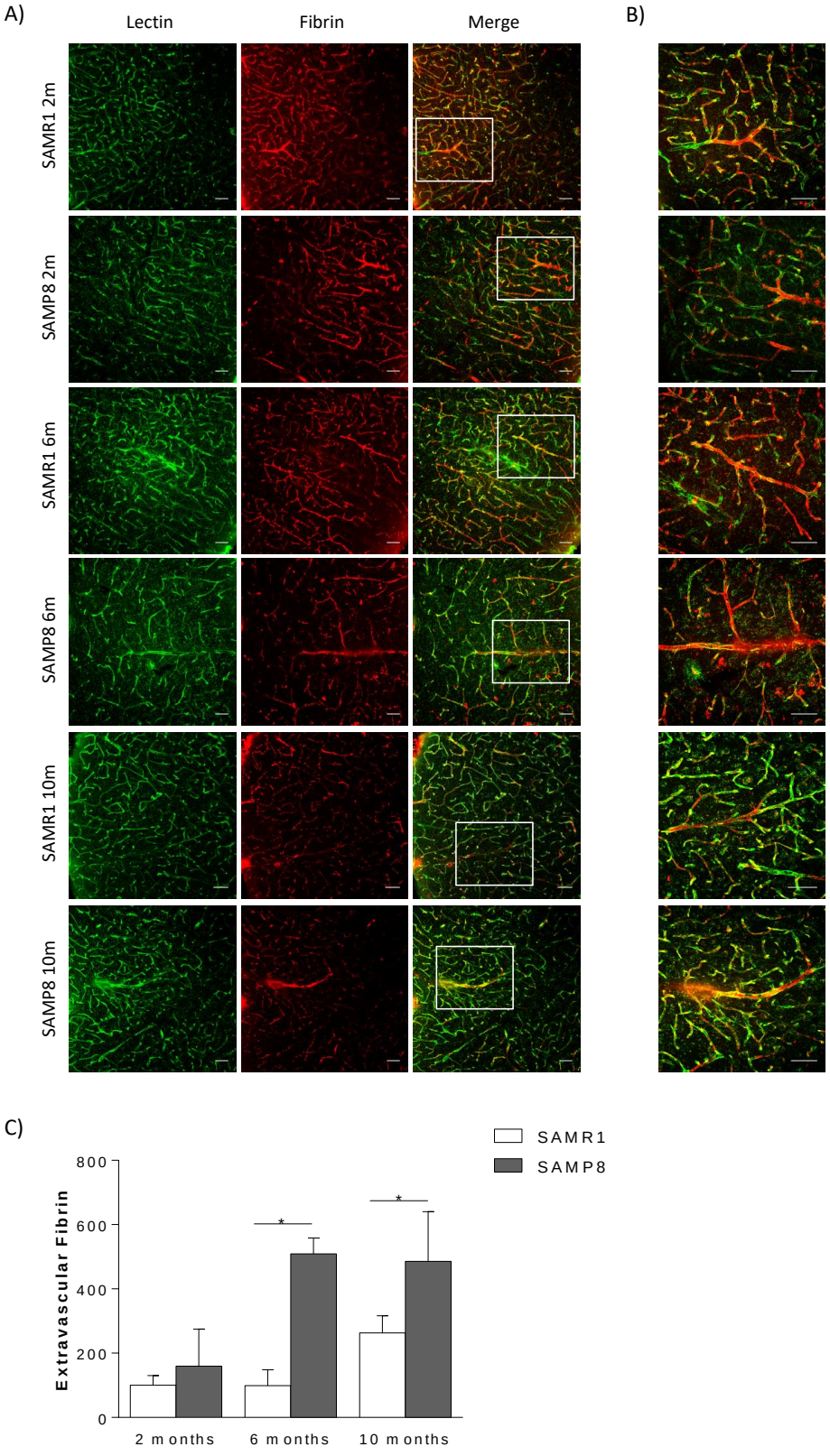


Figure 15. Effect of aging on BBB integrity using fibrin immunofluorescence. Representative confocal microscopy analysis of A) fibrin (red) and lectin-positive capillaries (green) on cortical brain sections and B) amplification of selected areas. In panel C) quantification of the extravascular fibrin. * Main effect of strain, two way ANOVA, $p<0.05$. Scale bar, 100 μm.

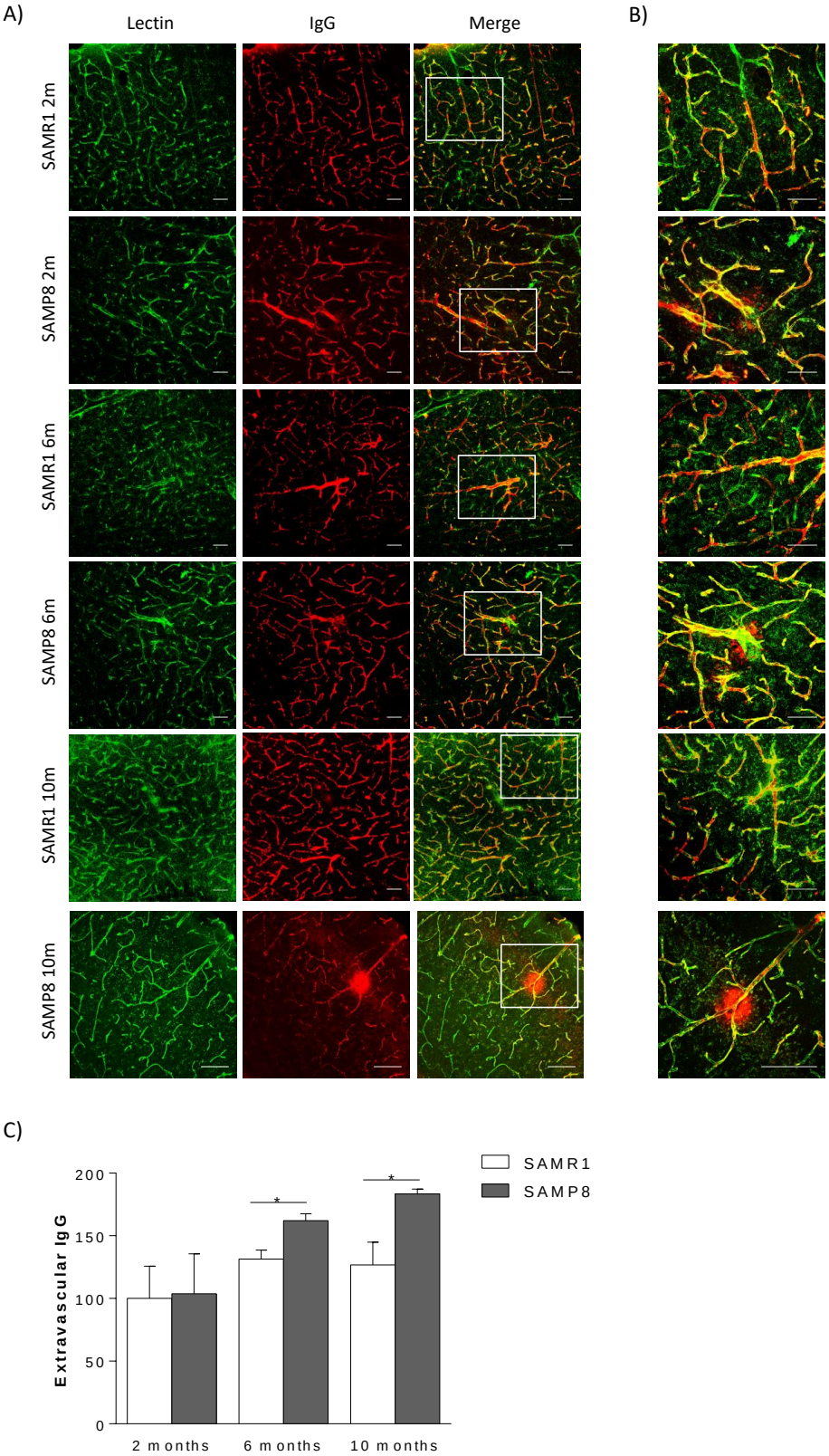


Figure 16. Effect of aging on BBB integrity using IgG immunofluorescence. Representative confocal microscopy analysis of A) IgG (red) and lectin-positive capillaries (green) on cortical brain sections and B) amplification of selected areas. In panel C) quantification of the extravascular fibrin. *Main effect of strain, two way ANOVA, $p<0.05$. Scale bar, 100 μm.

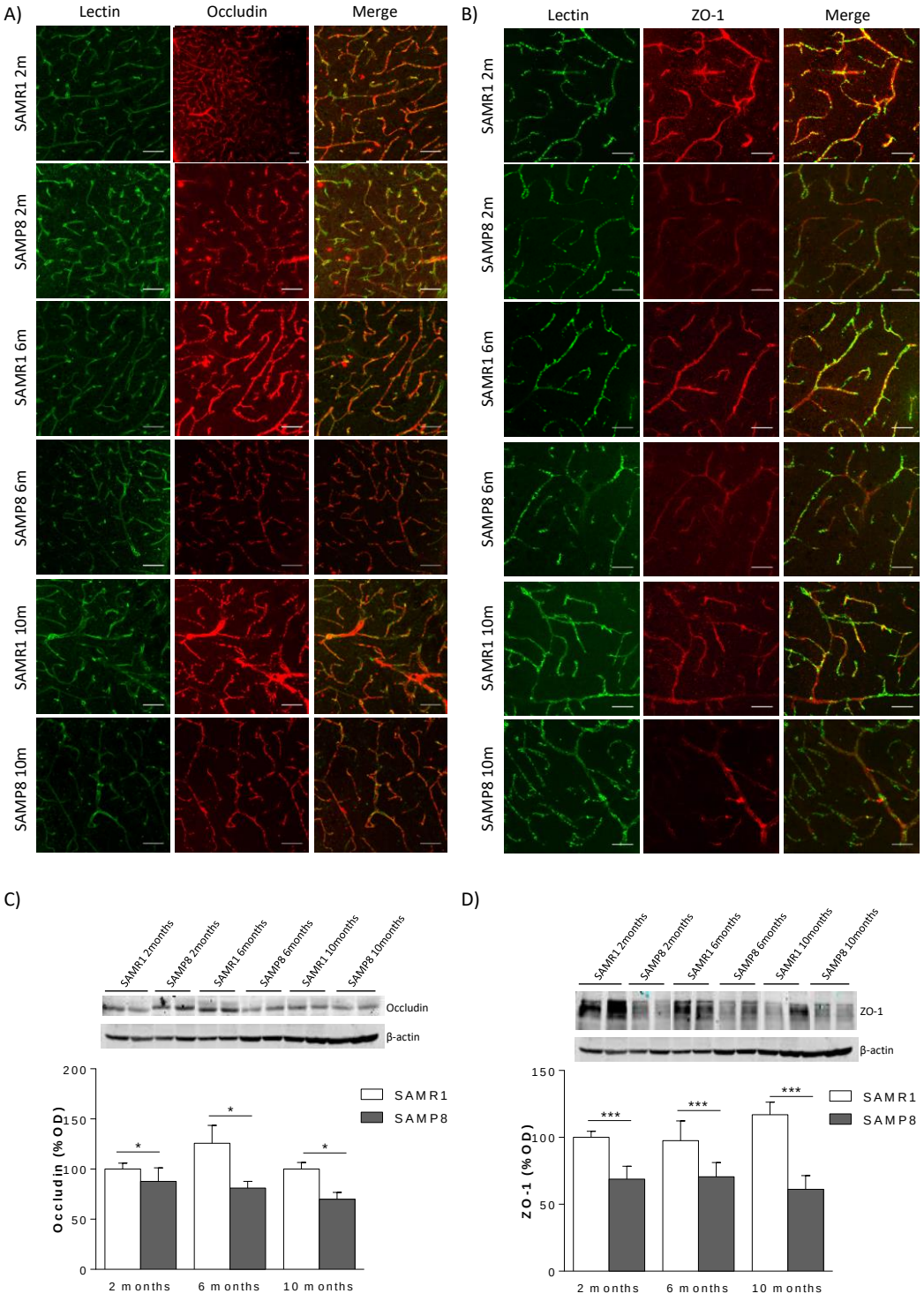


Figure 17. Effect of aging on BBB tight junctions. Representative confocal microscopy analysis of A) occludin (red) and B) ZO-1 (red) and lectin-positive capillaries (green) on cortical brain sections. In panel C) Occludin and D) ZO-1 representative immunoblots from mice brain tissue and their quantification. * Main effect of strain, two way ANOVA, $p<0.05$. Scale bar, 100 µm.

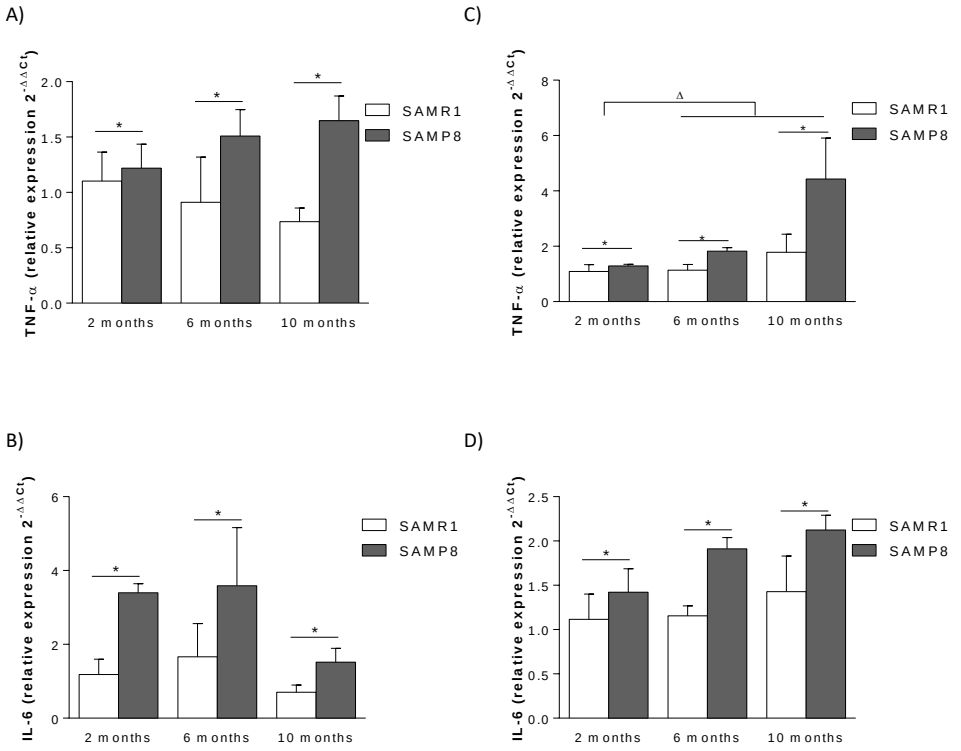


Figure 18. Effect of aging on proinflamamtory cytokine release. Peripheral (white adipose tissue) A) TNFα and B) IL-6 gene expression. Cortical (frontal cortex) C) TNF-α and D) IL-6 gene expression. *Main effect of strain, two way ANOVA; $^{\Delta}$Main effect of age, two way ANOVA.

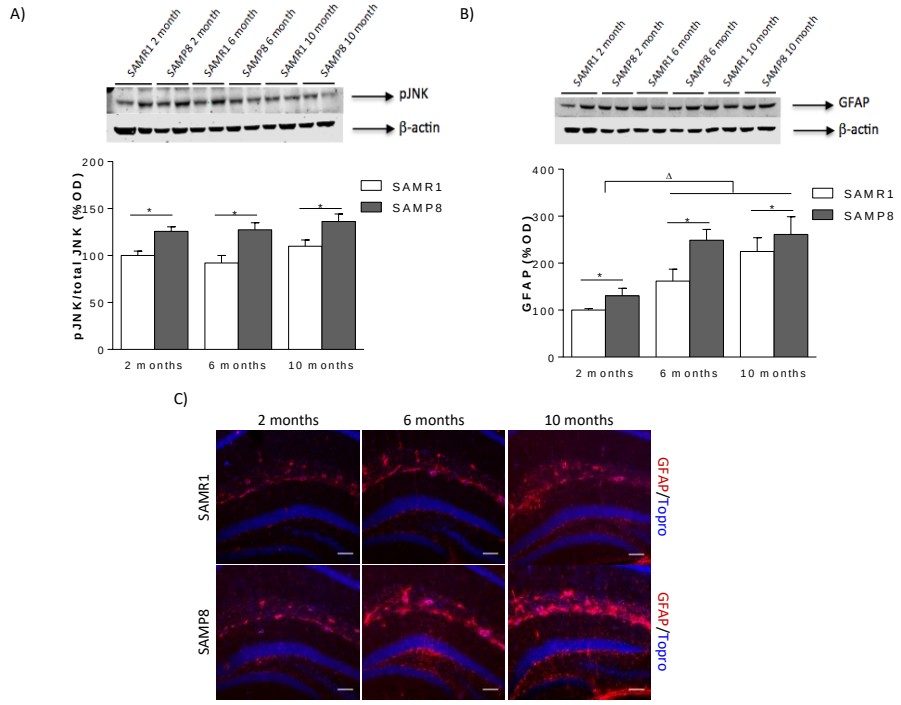


Figure 19. Effect of aging on neuroinflammation. Panel A) p-JNK protein expression level. Panel B) GFAP protein expression levels. Panel C) GFAP immunohistochemical representative images. Scale bar 100 µm. Figures A and B show percentage of optical density (O.D.) values of control 2 months SAMR1 mice and representative picture of the blotting. *Main effect of strain, two way ANOVA; $^{\Delta}$Main effect of treatment, two way ANOVA.

2.5 Aging induces gut microbiota dysbiosis

α-diversity is a measure of microbiome diversity within a community and is mainly concerned with the number of different bacteria or species therein. Shannon and Simpson are indices commonly used to evaluate α-diversity of microbiota. Shannon (p=0.012904, Figure 20A) and Simpson (p=0.041409, Figure 20B) indices were significantly lower in fecal samples from SAMP8 mice, thus indicating less diversity of bacteria. On the other hand, β-diversity is an estimate of similarity or dissimilarity between populations. PCoa-Jensen Shannon Divergence analysis revealed that dots from SAMR1 mice were not close to those of SAMP8 (R=0.333, p<0.05), suggesting that SAMP8 have a different microbiome composition than SAMR1 (Figure 20C).

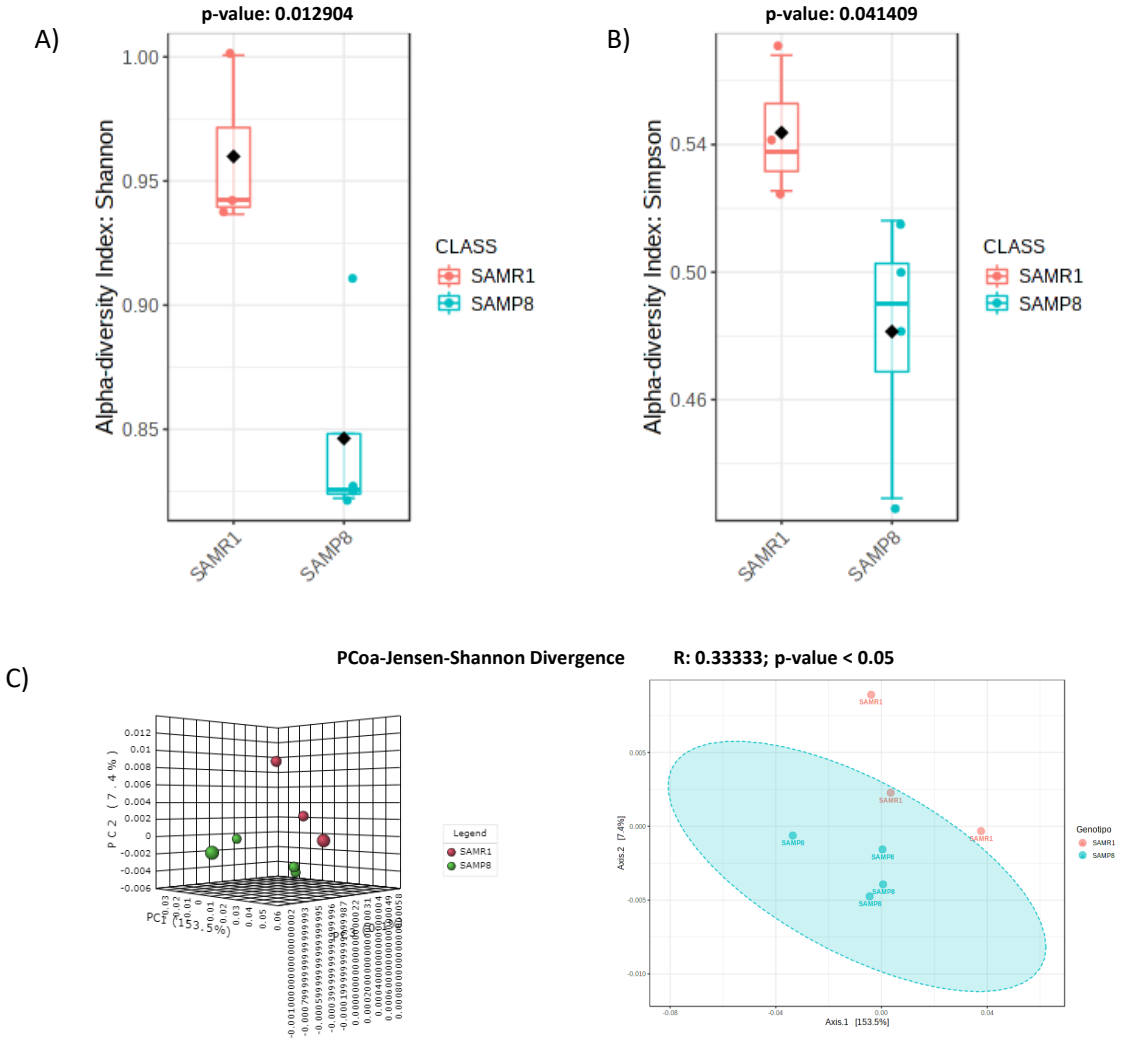


Figure 20. Differential profiles of the gut microbiota between SAMR1 and SAMP8 mice. A) Shannon index (*t*-test, $P < 0.05$). B) Simpson index (*t*-test, $P < 0.05$). C) PCoA analysis of gut bacteria data (Jensen-Shannon Divergence). Analysis of group similarities (ANOSIM)

Aging induced a marked alteration in gut dysbiosis observed by the changes in phylum abundance (Figure 21). Specifically, SAMP8 mice showed an increase in Bacteroidetes phylum (Figure 22A) that was probably due to an increase in *Prevotella* genus (Figure 22B). Moreover, a profound decrease in Proteobacteria phylum was found (Figure 22C), with a marked decrease in Deltaproteobacteria class (Figure 22D). Although no changes were observed in the Firmicutes phylum (Figure 22E), it is worth mentioning a marked decrease in *Dorea* genus (Figure 22F). Finally, although the Actinobacteria phylum appeared unchanged in SAMP8 mice (Figure 22G), Actinobacteria class showed a significant increase (Figure 22H).

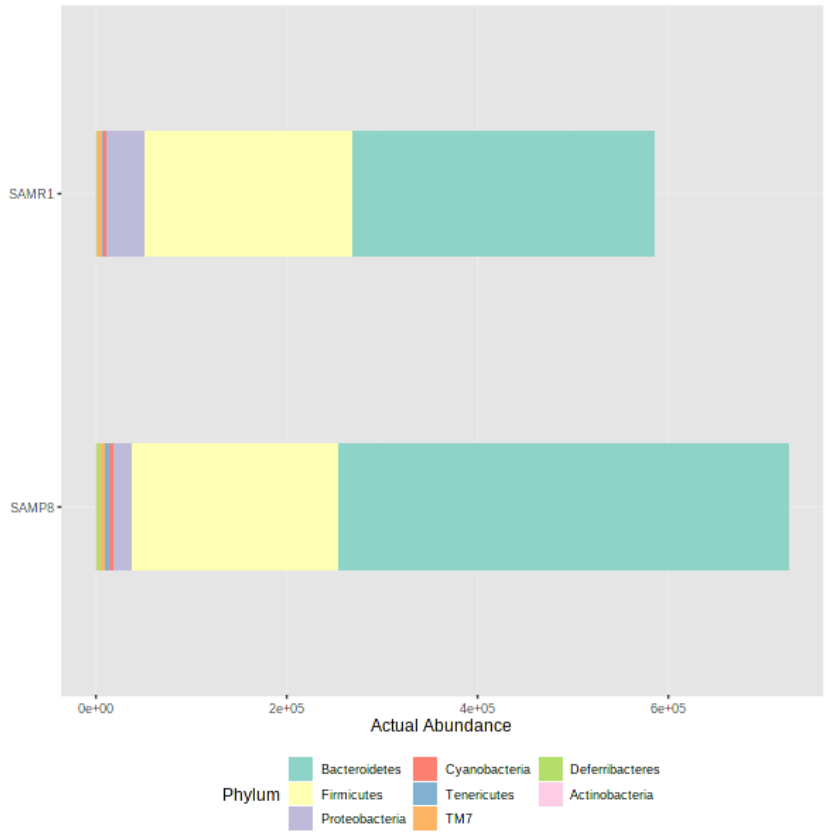


Figure 21. Effect of aging on gut dysbiosis. Bacterial phylum distribution in SAMR1 and SAMP8 mice.

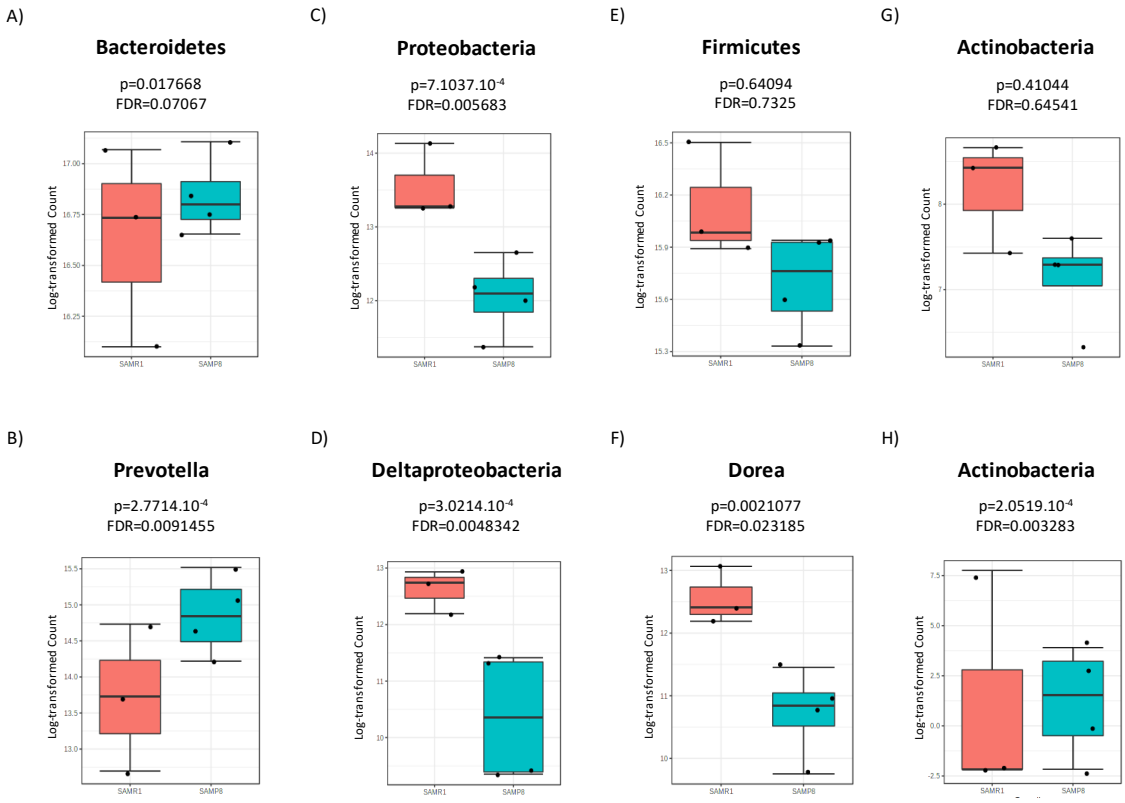


Figure 22. Effect of aging on gut dysbiosis. Bacterial phylum, class and genera that are significantly changed (edgeR p value <0.05 and FDR <0.05) between SAMPR1 and SAMP8 mice groups. Data were log-transformed counts of bacterial 16 S rRNA gene copies.

2.6 Aging increases peripheral and central TMAO levels

As depicted in figure 23A, aging induces a significant increase in serum TMAO levels of SAMP8 mice (two way ANOVA, $F_{2,30}$=9.878; p<0.001 followed by Tukey's p<0.05 SAMP8 vs rest of the groups). Interestingly, elevated levels of TMAO are also observed in the aging brain tissue of SAMP8 mice (two way ANOVA, main effect of age $F_{2,31}$=62.43; p<0.0001) (Figure 23B). In the same line, human CSF TMAO levels showed a positive correlation with aging (Spearman r=0.5793, p<0.01) (Figure 23C) and BBB integrity measured by brain albumin quantity (Spearman r=0.6740, p<0.01) (Figure 23D).

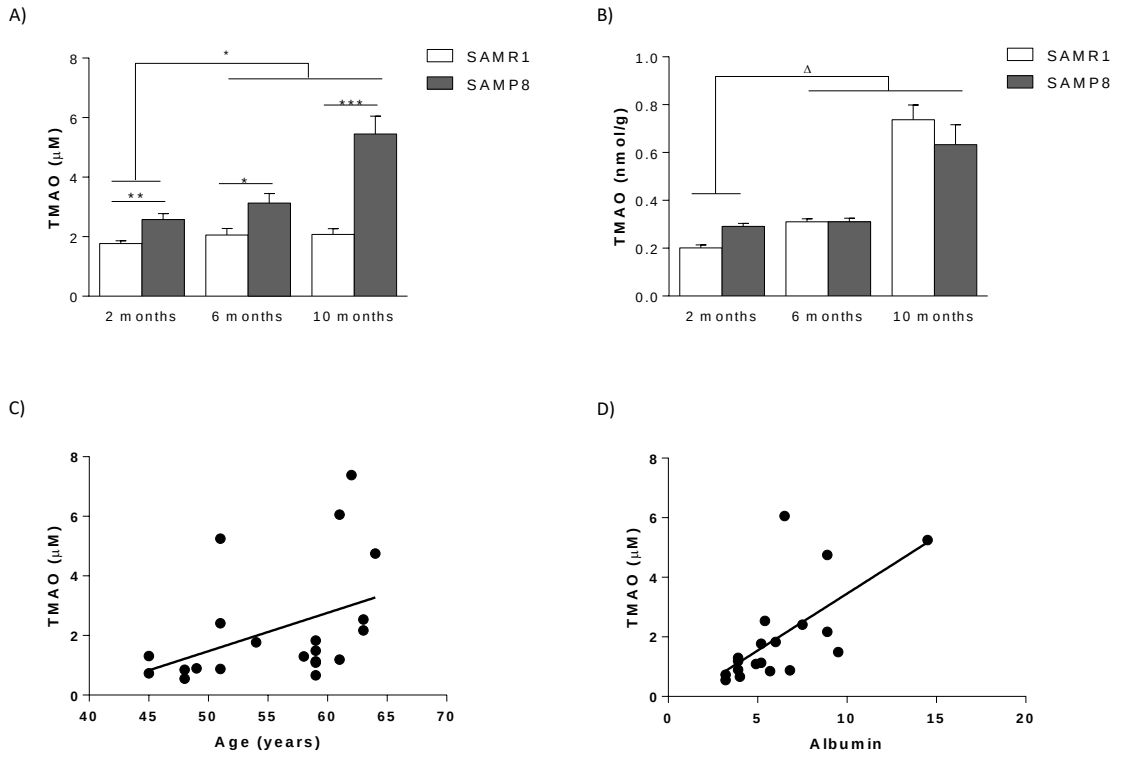


Figure 23. Effect of aging on TMAO levels. In panel A) TMAO levels in plasma samples in mice and B) TMAO levels in mouse brain samples. Human CSF TMAO levels correlate with age (panel C) and BBB integrity (panel D).

2.7 DMB treatment improves peripheral status in SAMP8 aged 6 months

Treatment with DMB, choline TMA lyase enzyme inhibitor that decreases TMAO plasma levels, showed clear improvement of peripheral metabolism, as SAMP8 mice treated with DMB showed reduced adiposity (two way ANOVA, main effect of treatment $F_{1,38}$=6.449; p<0.05) (Figure 24A), improved glucose tolerance (repeated measures ANOVA, $F_{12,124}$= 2.567; p<0.01) (Figure 24B), lowered fasting plasma glucose (two way ANOVA, main effect of treatment $F_{1,26}$=12.99; p<0.01) (Figure 24C) and insulin levels (two way ANOVA, main effect of treatment $F_{1,18}$=7.444; p<0.01) (Figure 24D), that leads to a decreased HOMA index (two way ANOVA, main effect of treatment $F_{1,18}$=37.13; p<0.0001, main effect of genotype $F_{1,18}$=8.681;p<0.01) (Figure 24E).

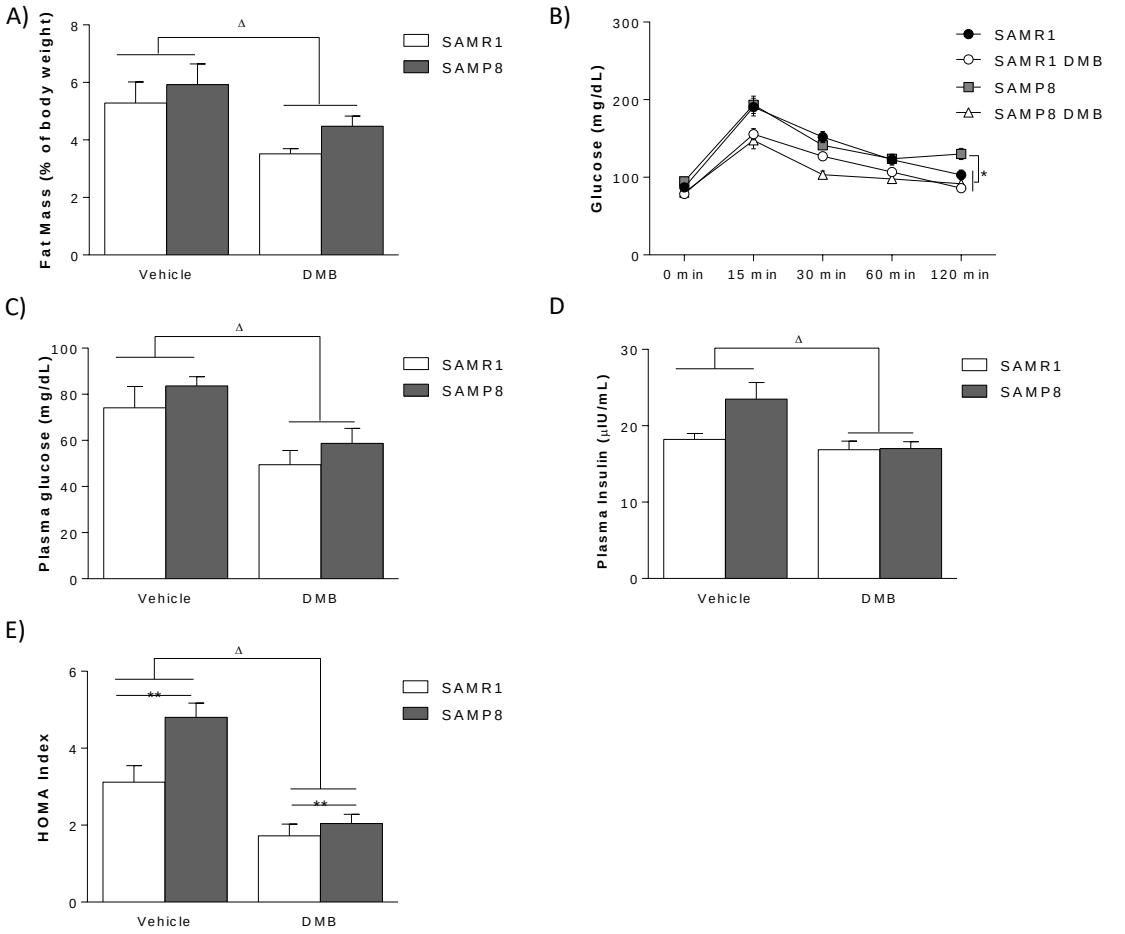


Figure 24. Effect of DMB on peripheral status. In panel A) mice adiposity measurement. B) Glucose tolerance tests (GTT) after treatment with DBM. C) Blood glucose levels, D) Blood insulin levels and E) Homeostatic model assessment indices of insulin resistance (HOMA) after treatment with DBM. *Main effect of strain, two way ANOVA; $^{\Delta}$ Main effect of treatment, two way ANOVA.

2.8 DMB treatment restores neuroinflammation and brain gliosis

SAMP8 mice showed higher expression of proinflammatory markers in hippocampus. However, DMB treated mice showed a decrease in brain neuroinflammatory markers, i.e., lower TNFα (two way ANOVA, main effect of treatment $F_{1,27}$=2.887; p<0.05, main effect of treatment $F_{1,27}$=4.891; p<0.05) (Figure 25A), p-eNOS at Thr-495 which is an inhibitory site (two way ANOVA, main effect of treatment $F_{1,18}$=4.522; p<0.05, main effect of genotype $F_{1,18}$=6.388; p<0.05) (Figure 25B), p-JNK (two way ANOVA, main effect of treatment $F_{1,24}$=6.462; p<0.05) (Figure 25C) and GFAP levels (two way ANOVA followed by Tukey's p<0.05 SAMP8 vs SAMR1 vehicle) (Figure 25D).

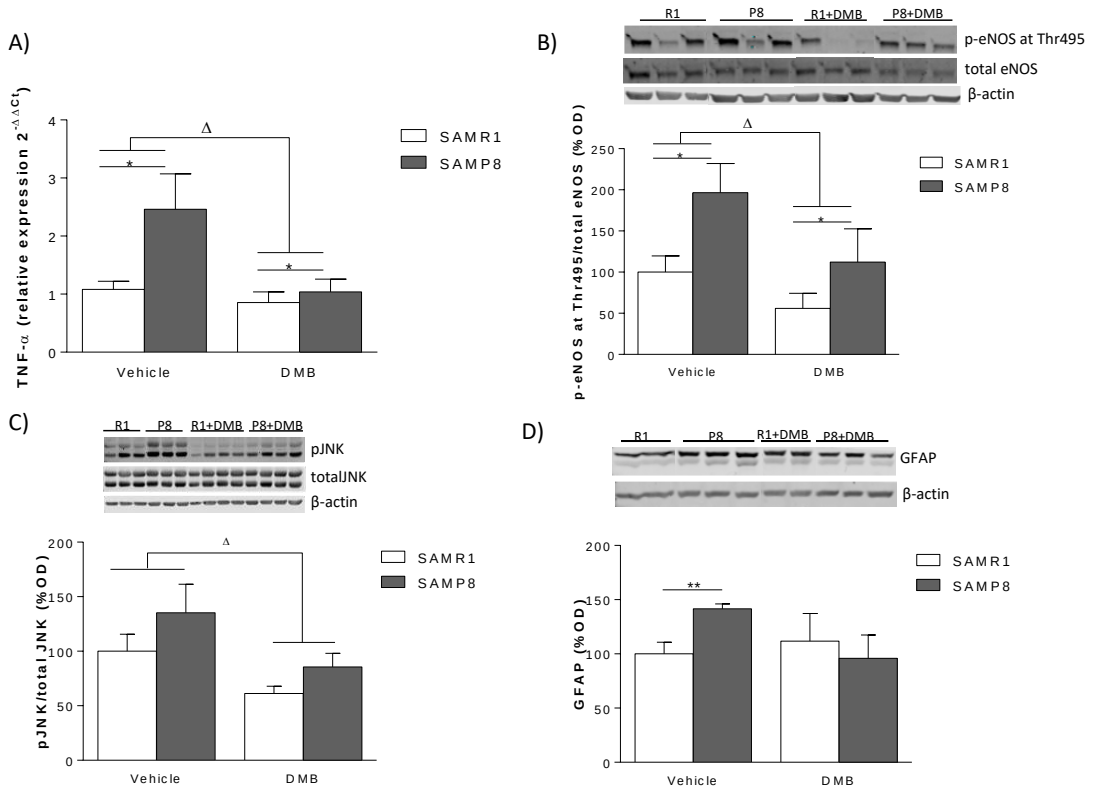


Figure 25. Effect of DMB on neuroinflammation. Hippocampus. In panel A) TNF-α expression, B) p-JNK protein levels, C) GFAP protein levels and D) p-eNOS protein levels at the inhibitory site Thr-495. *Main effect of strain, two way ANOVA; $^{\Delta}$ Main effect of treatment, two way ANOVA.

2.9 DMB treatment improved performance during behavioral tests

DMB treated SAMP8 mice showed a significantly improved performance in the acquisition Morris water maze test (repeated measures ANOVA, $F_{3,29}$= 24.80; p<0.0001) (Figure 26A) as well as in the second probe trial (two way ANOVA, $F_{1,23}$=6.644; p<0.05, followed by Tukey, p<0.05 SAMP8 vehicle vs SAMR1 vehicle) (Figure 26B).

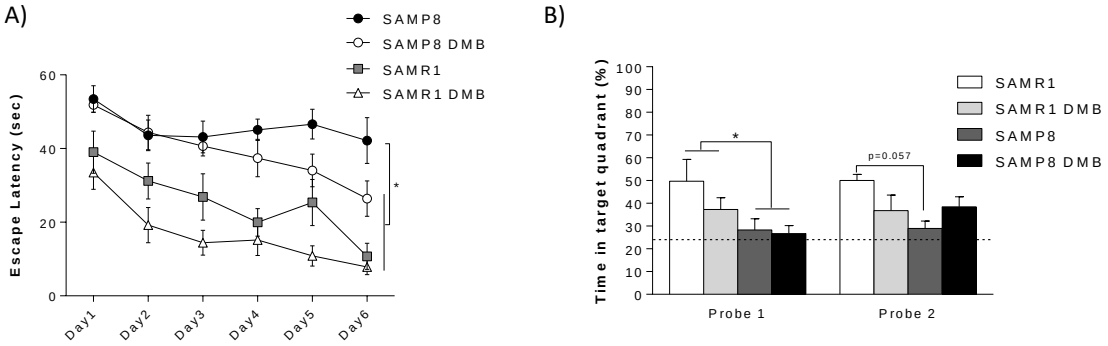


Figure 26. Effect of DMB in cognition. In panel**:** Cognitive performance assessed by Morris water maze (MWM) A) acquisition phase and B) retention phase. *Main effect of strain, two way ANOVA; $^{\Delta}$ Main effect of treatment, two way ANOVA.

DISCUSSION

DISCUSSION

In recent decades, significant advances in modern medicine, coupled with an improved quality of life in developing countries, have significantly increased life expectancy and older people are expected to live even longer in the future (2). The improvement in life expectancy is inevitably accompanied by a subsequent escalation in the prevalence of age-related diseases. Among them, insulin resistance and neurodegenerative diseases such as AD are considered part of the main threats to health in old age (216,217).

Classically, insulin resistance and neurodegeneration have been considered unrelated pathological entities, either as a metabolic disorder that mainly affects glucose homeostasis in peripheral organs such as the skeletal muscle, liver and fat, or as a degenerative disease of the CNS, respectively. However, recent studies have raised the possibility that these diseases may have similar molecular roots. Indeed, recently, both diseases have been associated with impaired action of insulin in the CNS. This notion is supported by epidemiological studies that have found a link between insulin resistance and AD (218). However, other studies have not been able to reveal this relationship (218). Post-mortem analyzes of the brains of AD patients have shown that insulin receptors are downregulated (219), as observed during aging (220,221). This led to the hypothesis that neuronal insulin resistance may contribute to the etiology of AD. Therefore, it is believed that the close correlation between metabolic disturbances (such as diabetes mellitus) and cognitive deficits is mainly due to the establishment of insulin resistance and the associated changes in the pleiotropic effects of insulin on central physiological functions. Therefore, the aim of the present study was to investigate the missing link between aging, insulin resistance and dementia.

Aging is associated with marked alterations in insulin secretion, which commonly leads to hyperinsulinemia (220). In our hands, marked insulin impairment and glucose intolerance that worsen with the age was found in SAMP8 mice group. This together with a strong tendency of increased HOMA index indicates the existence of age related peripheral insulin resistance and altered glucose homeostasis. The critical involvement of abnormal insulin signaling in the etiology of metabolic diseases has been emphasized by the onset of mild obesity and altered glucose metabolism in neuronal insulin receptor knock out mice (222). Although the exact molecular mechanisms driving the onset of insulin resistance are not yet fully understood, it is evident that two of the main risk factors for development of insulin resistance are overweight/obesity and aging. Therefore, next step was to analyze if aging per se induces body weight changes and fat accumulation that could lead to peripheral insulin resistance. Body weight was increased equally over time in both strains studied in the present work but it was always lower in SAMP8 mice compared to SAMR1 due to their smaller size. Surprisingly, as aging induces fat content accumulation a higher fat mass was expected in SAMP8 mice; however, no differences in body composition were found between groups. Therefore, the observed insulin resistance is not related to a higher adiposity, which suggests that

an alteration in the central nervous system driven insulin signaling regulation could be responsible of this defect.

Interestingly, in parallel with the observed peripheral insulin resistance, SAMP8 mice showed a marked age dependent cognitive decline, at least in the Morris water maze, a hippocampal-dependent task. The results found on the NORT, where only 10 months old SAMP8 mice exhibited cognitive impairment in the 1 hour task together with the fact that the hippocampus is only minimally involved in memory for objects (223), would support the notion that memory deficits in aging firstly has its anatomical substrate on the hippocampus and only when elderly is reached cortex starts been affected. It is worth mentioning that even though 6 and 10 months mice seem to have a decreased activity in the open field test, other measurements of locomotor activity, such as swim speed in the Morris water maze or total exploration time in the first exposure to objects in the NORT, did not differ among groups.

Almost a century after the discovery of insulin as a peptide secreted by the pancreas (224), the classical view of insulin as one of the main regulators of glucose homeostasis by promoting glucose uptake in peripheral tissues, such as the muscle and fat pads, and suppressing hepatic glucose production has expanded to a wide range of physiological and cellular effects, including its neuroprotective function and the regulation of learning and memory (225). Based on this idea, we next studied if changes in insulin signaling in SAMP8 mice observed in the periphery were also occurring in the CNS and could be responsible of the cognitive deficits. Previous studies have shown that insulin sensitivity in the CNS is regulated in an age dependent manner. As we age, many of the steps that control insulin action change, from alterations in the level of insulin itself to its intracellular signaling pathways. In fact, in addition to the gradual generalized loss of basic physiological functions with age, the decrease in insulin action appears to be an inevitable consequence of aging (226). The expression of insulin receptors in the brain is also subjected to an age-dependent decline (227). Notably, the level of insulin receptor mRNA in the hypothalamus, cortex, and hippocampus of old rats is drastically reduced (227). Surprisingly, our data showed a slight decrease in pIRS1 and pAkt levels in 10 months SAMP8 mice without alteration in any other component of the brain insulin signaling. Thus, we can conclude that the peripheral insulin resistance observed in SAMP8 mice is not transferred to the CNS and therefore the cognitive decline seen in those mice may not be due to central insulin resistance.

Given that central insulin resistance does not seem to be the cause of the observed cognitive disturbances, one can hypothesize the involvement of another molecular mechanism that, on one hand, leads to peripheral insulin resistance and, on the other hand, contributes to cognitive deficiencies, thus explaining the clinical link between peripheral metabolic disturbances and neurodegenerative disorders. In an attempt to find that missing mechanism underlying the age related cognitive deficiency observed in SAMP8 mice in the present study, we investigated other mechanisms that could be related to insulin signaling and could be the cause of a cognitive alteration. Mounting evidence has shown that the existing association between insulin signaling and the low-

grade inflammatory tone observed in obesity may be one of the leading processes toward the onset of neuronal insulin resistance (228–231). Inflammation has been associated mainly with the release of cytokines, particularly TNF-α and IL-6. TNF-α activates inflammatory kinases such as c-Jun N-terminal kinase (JNK) (232–234). In addition to the detrimental effect of low-grade inflammation per se on cellular physiology, activation of inflammatory cascades blunt insulin receptor signaling directly, notably by interfering with the phosphorylation events downstream of insulin receptor activation (228). Specifically, activation of JNK leads to inhibition of IRS phosphorylation, and therefore to desensitization of insulin action (228,235). Interestingly, these aforementioned inflammatory pathways are also enhanced during normal aging (236,237). In our hands, a marked peripheral inflammatory state (i.e. significantly higher adipose tissue TNF-α and IL-6 levels) is observed in SAMP8 mice. The peripheral inflammation is accompanied by a strong neuroinflammation as SAMP8 mice exhibited significant increases of cortical TNF-α and IL-6 levels together with an elevated p-JNK levels as well as GFAP protein and inmmunoreactivity, already at 2 months of age. These results fit with the peripheral metabolism alterations and the cognitive deficiency observed in SAMP8 mice, suggesting that inflammation could be underlying both pathologies. Noteworthy, BBB integrity can be disrupted by neuroinflammation associated with many age-related disorders as AD (238). Indeed, SAMP8 mice showed elevated levels of brain fibrin and IgG accompanied by lower tight junction proteins expression, indicating an exacerbated BBB leaking. Thus, the aberrant amounts of peripheral proinflamamtory cytokines observed may enter the brain via the BBB and cause inflammation by altering glial activation (171,172) and subsequent brain cytokine release, leading to cognitive deficiencies.

Aging is a known risk factor for dysbiosis as it can alter gut microbiome composition and reduce microbiota diversity (132,139). Dysbiosis can lead to a major production of certain gut metabolites such as TMA, which proceeds from the bacterial synthesis from substrates like choline or L-carnitine. It is then rapidly absorbed and further oxidized by hepatic enzymes (FMO1 and FMO3) to form TMAO (50). In our hands, a profound decrease in Proteobacteria, with a marked increase of Bacteroidetes was observed in SAMP8 mice. Bacteroidetes and specifically, *Prevotella* genus that appear to be significantly increased in SAMP8 mice have been associated with higher TMAO production. Indeed, literature has shown that individuals with an enterotype characterized by enriched proportions of *Prevotella* have significantly higher plasma TMAO, than individuals with a *Bacteroides* enterotype, indicating that enterotypes affect the host (73). The higher TMAO levels could be also related to the observed decrease in the genus *Dorea*, as it has been previously reported in human samples where proportions of *Dorea* within recipient feces were inversely correlated with both plasma TMAO levels and atherosclerosis extent (239). Furthermore, in parallel to our data, TMAO has been extensively positively correlated with the genus Bifidobacterium (phylum Actinobacteria) (50,240), concluding that the observed increases in this class could also lead to higher TMAO production.

In the last few years, TMAO has been widely associated with inflammation, glucose impairments and even neuropathologies (50). HFHS diet intake affects the composition of microbiota and may contribute to an imbalanced microbial environment in the intestine. Therefore, it has been hypothesized recently that the gut microbiota may be part of a mechanistic connection between consumption of HFD and other unbalanced diets and impaired cognition. The literature has showed that short-term diet exposure is able to change microbiota composition in human (207) and mice (241) within days and these changes may be relevant to early onset of cognitive impairment (242). In fact, preclinical experiments have confirmed that HFD may change the gut microbiota and contribute to development of dementia (77,243–245). Interestingly, a recent study of microbiome-metabolome signatures in an AD mouse model fed with a normal or HFD have demonstrated that HFD and genetic predisposition to neurodegenerative disease share similar abnormalities in the gut microbiome (77). The study of serum and fecal metabolites of those mice revealed a deficiency in unsaturated fatty acids and choline, and an excess in ketone bodies, lactate, amino acids, TMA, and TMAO in AD mice fed with a HFD, which in turn are related to cognitive impairment and cerebral hypometabolism (50).

Based on those evidences and in order to better understand if TMAO could be the link between metabolic diseases and cognitive deficiencies, we studied the adipocyte (3T3-L1 cell line) cell viability upon a TMAO treatment. Surprisingly, cell viability seemed not to be negatively affected by TMAO. On the contrary, TMAO treatment seemed to show some type of benefit over control. This was further confirmed by a cell differentiation assay, where TMAO increased the differentiation of mature adipocytes from preadipocytes, thus favoring the increase of fat adipose tissue. These results are in line with other studies showing that TMAO levels are linked to adiposity and could increase the expression of scavenger receptors CD36 and SR-A1 in macrophages (44,191,246). These scavengers promote lipid accumulation and foam cell formation. Moreover, other studies have showed that FMO3 expression (the enzyme that converts TMA into TMAO) is closely related to fat adipose tissue and its inhibition leads to beiging of white adipose tissue and promotes resistance to obesity (247).

Macrophage infiltration into adipose tissue contributes to the increased expression of inflammatory cytokines in obesity. However, it remains to be investigated why macrophage infiltration is increased in adipose tissue and/or the signals that induce the release of pro-inflammatory cytokines. Several studies have revealed an increase in the expression of proinflamamtory cytokines when plasma TMAO levels are elevated. The study performed by Rohrmann et al. (2016) (47) described a link between low grade inflammation and plasma TMAO levels. When the concentration of plasma TMAO was augmented there was an overexpression of TNF-α, IL-6 and C-reactive protein. In our hands, TMAO 200 μM was able to increase the expression of pro-inflammatory cytokines IL-6, TNF-α and IL-1β. These results agree with the results showed previously by Geng G et al., (248), who showed a huge raise in protein and mRNA expression of IL-6, TNF-α and ICAM-1 in macrophages after TMAO treatment. The

concentration used for the experiments was carefully chosen by the cell viability assay, which demonstrated that the only concentration that affected cell viability was 1000 μM. Based on this evidence and on the concentration of TMAO used for in vitro experiments in the literature for other cell lines (46,48,49,108,246,248–250), all our experiments included the concentration of 200 μM as recent epidemiological studies had showed TMAO in plasma ranges widely from 0.08 to 250 μM (69).

TMAO has been suggested to cause BBB disruption by reducing the expression of tight junction proteins like claudin-5 and tight junction protein-1 (ZO-1), favoring its access to the brain (107,108). In our hands, TMAO concentrations that did not alter cell viability (under 1000 μM) were able to reduce the expression of tight junction proteins like ZO-1 and Occludin in HCMECs cells, thus disrupting the BBB. In this condition, aberrant amounts of plasma proinflamamtory cytokines and other detrimental molecules (such as TMAO *per se*) may enter the brain via the BBB and cause inflammation by altering microglial maturation (171) and astrocyte activation (172).

On the other hand, recent studies have suggested that TMAO can reach the brain and cross the BBB and/or the blood-CSF barrier (101,102). As TMAO seems able to cross BBB it may have direct contact with brain cells such us neuron, astrocytes and microglia. Cell viability of primary neuronal cultures was not affected by TMAO. However, it raised the expression of proinflamamtory cytokine IL-1β. This result is also in line with other evidences in the literature showing that NLRP3 inflammasome is a cytokine-activating protein complex of the IL-1β family (251) which has been widely linked to TMAO (46,48,49,108,160,251–253). Thus, NLRP3 formation and activation would cause a major expression and release of the proinflamamtory cytokine IL-1β (48,108) that would lead to neuroinflammation.

Neuroinflammation is mainly driven by activation of glial cells. Specifically, microglia plays a critical role in CNS immunomodulation as well as in clearance of dying neurons, pathogens and other substances such as unfolded proteins or cellular debris which is necessary for brain homeostasis (254). CD16/CD32 is a classic proinflamamtory marker located in the membrane of microglia and widely used to detect active microglia (255–257). In our experiments, expression of CD16/CD32 was increased even with the lowest concentration, showing that presence of TMAO activates microglia in a proinflamamtory manner.

Phagocytosis had been previously seen as a beneficial process for homeostasis preventing the spillover of neurotoxic and pro-inflammatory substances by clearing dying cells (258). However, nowadays the evidence suggests that microglia activation rely on different targets and receptors that finely control its responses. “Eat-me signals” play a crucial role in recognition of extracellular debris and initiation of phagocytosis; activating signaling cascades and phagocytic receptors trying to facilitate discriminative clearance of apoptotic cells (259). For example, phagocytosis of apoptotic neurons has been associated to decreased production of pro-inflammatory cytokines (260) while

myelin debris phagocytosis enhanced pro-inflammatory phenotype and dampened anti-inflammatory profile of microglia (261).

In the present study, after TMAO treatment, microglia showed higher rates of myelin phagocytosis, mainly with the highest concentrations. While transient neuroinflammation is beneficial to phagocyte pathogens and cell debris, chronically activated microglia acquire a phagocytic profile characterized by major release of cytotoxic and pro-inflammatory molecules such as TNF-α. Furthermore, excessive phagocytosis may also be harmful as it could lead to phagoptosis of harmed but viable neurons that may recover with time if they are not phagocytosed (256,262). Myelin phagocytosis is a process that has been linked to phagoptosis (256) so excessive myelin phagocytosis as we have found in our in vitro experiments could lead to phagoptosis of stressed but viable cells and promote degeneration processes.

In the light of the data obtained in our *in vitro* studies and according to the literature (155), in the present study, an age-dependent increase of TMAO levels were observed not only in the periphery but also in the CNS, probably directly linked to the higher BBB leaking observed in SAMP8 mice. Therefore, and based in all the data obtained *in vitro*, it is tempting to speculate that aging-induced dysbiosis can lead to higher TMAO levels inducing a systemic inflammatory status that may progress, in the context of a disrupted BBB, towards neuroinflammation, producing anomalous glial activation higher cytokine release and cognitive deficiencies in last instance (139). This hypothesis agrees with the data we found in our human samples, where TMAO correlated not only with aging but with the integrity of the BBB.

In this context, a pharmacological treatment with DMB was established to reduce TMAO levels and ameliorate the cognitive decline seen at 6 months, prior to the huge increase of serum TMAO levels in SAMP8 mice. DMB is an analogue of choline that is able to reduce most but not all TMA lyases. It inhibits the transformation of choline, carnitine and crotonobetaine into TMA although it is not able to avoid the conversion of GBB (191).

DMB was able to impact on peripheral status. Indeed, mice receiving DMB treatment showed a lower percentage of fat adipose tissue. This result is in concordance with our in vitro experiments where we found that TMAO increased the differentiation of preadipocytes to mature adipocytes. Moreover, DMB treatment was able to reverse peripheral glucose alterations and to decrease HOMA index in SAMP8 mice. DMB had a beneficial effect on glucose tolerance, improving the performance of both strains during GTT. Higher levels of glucose during GTT may be due to several factors such as impaired glucose utilization, a decrease in insulin secretion or even presence of insulin resistance. Fasting glucose levels prior to sacrifice of mice were lower in DMB treated animals too, what suggest an improvement in glucose utilization. SAMP8 also showed greater levels of fasting insulin that were restored to normal levels after DMB treatment. To sum up, we can conclude that DMB benefits in GTT may be due to greater peripheral uptake of glucose. Furthermore, we found lower insulin levels in serum of

DMB treated mice and better values for HOMA-IR index, which is an indicator of insulin resistance, thus insulin may have contributed to better glucose tolerance improving its uptake (263).

In parallel, cognitive performance was significantly improved as seen by SAMP8 mice performance in MWM. This could be linked to a marked decrease in inflammatory markers such as p-JNK and TNF-α in hippocampus were observed. Additionally, proinflammatory cytokines such as TNF-α are able to downregulate eNOS activity, reducing NO production and bioavailability, driving to endothelial dysfunction (264). Moreover, TMAO has been recently suggested to impair eNOS activity (71,197,192,200). There are multiple sites of phosphorylation involved in eNOS activity. One of them is phosphorylation at Thr495, which is a negative regulatory site linked to decreased enzyme activity (264). A recent study showed that mice with chronic kidney disease, which is a key regulator in TMAO levels, tended to an increase of this phosphorylation site (200). In our study, we found that SAMP8 mice showed a greater rate of phosphorylation at this site, thus presenting decreased enzyme activity, which was normalized with DMB treatment. This result is consistent with the fact that inflammaging have been related to a reduction of eNOS activity (265).

Thus, DMB may exert its beneficial effects by improving the peripheral status (i.e. better glucose tolerance and insulin signaling decreasing peripheral low grade inflammation), and restoring, in parallel, neuroinflammation.

The restoration of brain inflammation could be also directly linked to a decrease in brain gliosis. In this context, we demonstrated that DMB was able to restore levels of reactive microglia, which are known to mediate brain inflammation. It is remarkable to remind that in our *in vitro* experiments, the presence of TMAO was able to activate microglia and raise the expression of the pro-inflammatory marker CD16/CD32. Therefore, DMB treatment by decreasing TMAO levels could be able to inhibit microglial activation and subsequent proinflamamtory cytokine release, as observed in DMB treated mice. Furthermore, astrocytes are known to change their morphology and up-regulate some markers like GFAP when they undergo activation and mediate inflammation (266). Concretely, an enhancement of GFAP expression by astrocytes is known as astroglioisis which can be a beneficial due to the promotion of neuronal growth and survival, but it may also be damaging when continuous activation occurs (267). DMB treatment decreased GFAP levels indicative of a lower astrocyte activation.

Taken together, we can conclude that aging induced insulin resistance and dysbiosis with the subsequent elevated TMAO levels, can lead to an inflammatory state that could cause neuroinflammation on the one hand, and contribute to cognitive deficiency on the other. In this line, it is tempting to speculate that treatment with DMB could be a novel therapeutic strategy for the prevention and treatment not only for insulin resistance but also for aging-related cognitive decline.

CONCLUSIONS

CONCLUSIONS

The results obtained in the present Doctoral Thesis have led to the following conclusions:

1. Administration of TMAO to *in vitro* models was able to increase the differentiation of mature adipocytes from adipocytes, increase expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in macrophages and reduce expression of tight junction proteins in BBB cells.

2. TMAO was able to activate microglia and raise the expression of the pro-inflammatory marker CD16/CD32, increasing aberrantly myelin phagocytosis. On the other hand, TMAO also increased expression of pro-inflammatory cytokines IL-1β and IL-6 in neurons.

3. SAMP8 mice showed a marked insulin impairment and glucose intolerance that is aggravated with the age.

4. Peripheral insulin resistance observed in SAMP8 mice does not seem to be transferred to the CNS as only a decrease in pIRS1 and pAkt levels in 10 months SAMP8 mice without alteration in any other component of the brain insulin signaling were found. Therefore the cognitive decline seen in those mice does not seem to be due to the development of central insulin resistance.

5. SAMP8 mice showed a marked age dependent cognitive decline, evaluated with the NORT, a cortical dependent task, as well as, in the Morris water maze, a hippocampal-dependant test.

6. Aging disrupts BBB integrity in SAMP8 mice, assessed by higher fibrin and IgG leaking and decreased levels of tight junction proteins such as ZO-1 and Occludin. SAMP8 mice showed increased plasma and brain TMAO levels. In the same line, in humans, increased levels of TMAO in CSF were correlated with aging and BBB integrity.

7. SAMP8 mice showed increased TNF-α and IL-6 expression in white adipose tissue and cortex, indicative of peripheral and central inflammation. Neuroinflammation was further confirmed by significantly higher p-JNK levels as well as GFAP protein expression and inmmunoreactivity already in 2 months SAMP8 mice. These results suggest that neuroinflammation could be responsible for the cognitive impairment observed in those mice.

8. Aging induced gut dysbiosis, showing reduced diversity and different microbiota composition with enterotypes associated with greater TMAO levels.

9. Treatment with DMB had a positive impact on peripheral status reducing fat adipose content. Moreover, DMB reversed peripheral glucose alterations and insulin resistance in SAMP8 mice, improving GTT performance and HOMA index.

10. Finally, DMB treatment alleviated and restored cognitive impairment in SAMP8 mice as seen by SAMP8 mice performance in MWM acquisition and retention. This effect could be linked to a marked decrease in inflammatory markers such as p-JNK and TNF-α in hippocampus and the reduced inhibition of eNOS. The restoration of brain inflammation could be directly linked to a decrease in brain gliosis as DMB restored GFAP levels in SAMP8 mice.

Taken together, we can conclude that aging induced insulin resistance and dysbiosis with the subsequent elevated TMAO levels, can lead to an inflammatory state that could cause neuroinflammation on the one hand, and contribute to cognitive deficiency on the other. In this line, it is tempting to speculate that treatment with DMB could be a novel therapeutic strategy for the prevention and treatment not only for insulin resistance but also for age-related cognitive decline.

REFERENCES

REFERENCES

1. González-Muniesa P, Mártinez-González MA, Hu FB, Després JP, Matsuzawa Y, Loos RJF, et al. Obesity. Nat Rev Dis Prim. 2017;15;3:17034.

2. WHO. WHO. World Health Organization (WHO): Obesity and overweight. World Health Organization. 2020.

3. Profenno LA, Porsteinsson AP, Faraone S V. Meta-Analysis of Alzheimer's Disease Risk with Obesity, Diabetes, and Related Disorders. Biol Psychiatry. 2010;15;67(6):505-12.

4. Sabia S, Kivimaki M, Shipley MJ, Marmot MG, Singh-Manoux A. Body mass index over the adult life course and cognition in late midlife: The Whitehall II Cohort Study. Am J Clin Nutr. 2009;89(2):601-7.

5. Elias MF, Elias PK, Sullivan LM, Wolf PA, D'Agostino RB. Obesity, diabetes and cognitive deficit: The Framingham Heart Study. Neurobiol Aging. 2005;26 Suppl 1:11-6.

6. Cournot M, Marquié JC, Ansiau D, Martinaud C, Fonds H, Ferrières J, et al. Relation between body mass index and cognitive function in healthy middle-aged men and women. Neurology. 2006;67(7):1208-14.

7. Enzinger C, Fazekas F, Matthews PM, Ropele S, Schmidt H, Smith S, et al. Risk factors for progression of brain atrophy in aging: Six-year follow-up of normal subjects. Neurology. 2005;64(10):1704-11.

8. Ward MA, Carlsson CM, Trivedi MA, Sager MA, Johnson SC. The effect of body mass index on global brain volume in middle-aged adults: A cross sectional study. BMC Neurol. 2005;5:23.

9. Gorospe EC, Dave JK. The risk of dementia with increased body mass index. Age and Ageing. 2007;36(1):23-9.

10. Beydoun MA, Beydoun HA, Wang Y. Obesity and central obesity as risk factors for incident dementia and its subtypes: A systematic review and meta-analysis. Obesity Reviews. 2008;9(3):204-18.

11. Anstey KJ, Cherbuin N, Budge M, Young J. Body mass index in midlife and late-life as a risk factor for dementia: A meta-analysis of prospective studies. Obes Rev. 2011;12(5):e426-37.

12. Beilharz JE, Maniam J, Morris MJ. Diet-induced cognitive deficits: The role of fat and sugar, potential mechanisms and nutritional interventions. Nutrients. 2015;7(8):6719-38.

13. Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science. 2005;307(5717):1915-20.

14. Rhee SH, Pothoulakis C, Mayer EA. Principles and clinical implications of the brain-gut-enteric microbiota axis. Nature Reviews Gastroenterology and Hepatology. 2009;6(5):306-14.

15. Desbonnet L, Clarke G, Shanahan F, Dinan TG, Cryan JF. Microbiota is essential for social development in the mouse. Molecular Psychiatry. 2014;19(2):146-8.

16. Neufeld KM, Kang N, Bienenstock J, Foster JA. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol Motil. 2011;23(3):255-64, e119.

17. Rogers GB, Keating DJ, Young RL, Wong ML, Licinio J, Wesselingh S. From gut dysbiosis to altered brain function and mental illness: Mechanisms and pathways. Molecular Psychiatry. 2016;21(6):738-48.

18. Turnbaugh PJ, Bäckhed F, Fulton L, Gordon JI. Diet-Induced Obesity Is Linked to Marked but Reversible Alterations in the Mouse Distal Gut Microbiome. Cell Host Microbe. 2008;3(4):213-23.

19. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A. 2010;107(33):14691-6.

20. Magnusson KR, Hauck L, Jeffrey BM, Elias V, Humphrey A, Nath R, et al. Relationships between diet-related changes in the gut microbiome and cognitive flexibility. Neuroscience. 2015;300:128-40.

21. Gregor MF, Hotamisligil GS. Inflammatory mechanisms in obesity. Annu Rev Immunol. 2011;29:415-45.

22. Milagro FI, Mansego ML, De Miguel C, Martínez JA. Dietary factors, epigenetic modifications and obesity outcomes: Progresses and perspectives. Molecular Aspects of Medicine. 2013;34(4):782-812.

23. Cancello R, Clément K. Is obesity an inflammatory illness? Role of low-grade inflammation and macrophage infiltration in human white adipose tissue. BJOG: An International Journal of Obstetrics and Gynaecology. 2006;113(10):1141-7.

24. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes. 2008;57(6):1470-81.

25. Kanneganti TD, Dixit VD. Immunological complications of obesity. Nature Immunology. 2012;13(8):707-12.

26. Shu CJ, Benoist C, Mathis D. The immune system's involvement in obesity-driven type 2 diabetes. Seminars in Immunology. 2012;24(6):436-42.

27. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest. 2003;112(12):1796-808.

28. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-α in human obesity and insulin resistance. J Clin Invest. 1995;95(5):2409-15.

29. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. Protection from obesity-induced insulin resistance in mice lacking TNF- α function. Nature. 1997 ;389(6651):610-4.

30. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. Nature Reviews Immunology. 2011;11(2):85-97.

31. Wellen KE, Hotamisligil GS. Obesity-induced inflammatory changes in adipose tissue. Journal of Clinical Investigation. 2003;112(12):1785-8.

32. Sellbom KS, Gunstad J. Cognitive function and decline in obesity. Journal of Alzheimer's Disease. 2012;30 Suppl 2:S89-95.

33. Capuron L, Su S, Miller AH, Bremner JD, Goldberg J, Vogt GJ, et al. Depressive Symptoms and Metabolic Syndrome: Is Inflammation the Underlying Link? Biol Psychiatry. 2008;64(10):896-900.

34. Emery CF, Fondow MDM, Schneider CM, Christofi FL, Hunt C, Busby AK, et al. Gastric bypass surgery is associated with reduced inflammation and less depression: A preliminary investigation. Obes Surg. 2007;17(6):759-63.

35. Dinel AL, André C, Aubert A, Ferreira G, Layé S, Castanon N. Lipopolysaccharide-induced brain activation of the indoleamine 2,3-dioxygenase and depressive-like behavior are impaired in a mouse model of metabolic syndrome. Psychoneuroendocrinology. 2014;40:48-59.

36. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci. 2008;9(1):46-56.

37. Miller AA, Spencer SJ. Obesity and neuroinflammation: A pathway to cognitive impairment. Brain Behav Immun. 2014;42:10-21.

38. Jeon BT, Jeong EA, Shin HJ, Lee Y, Lee DH, Kim HJ, et al. Resveratrol attenuates obesity-associated peripheral and central inflammation and improves memory deficit in mice fed a high-fat diet. Diabetes. 2012;61(6):1444-54.

39. Puig KL, Floden AM, Adhikari R, Golovko MY, Combs CK. Amyloid precursor protein and proinflammatory changes are regulated in brain and adipose tissue in a murine model of high fat diet-induced obesity. PLoS One. 2012;7(1):e30378.

40. Pepping JK, Freeman LR, Gupta S, Keller JN, Bruce-Keller AJ. NOX2 deficiency attenuates markers of adiposopathy and brain injury induced by high-fat diet. Am J Physiol - Endocrinol Metab. 2013;304(4):E392-404.

41. Letra L, Santana I, Seiça R. Obesity as a risk factor for Alzheimer's disease: the role of adipocytokines. Metabolic brain disease. 2014;29(3):563-8.

42. Aguilar-Valles A, Inoue W, Rummel C, Luheshi GN. Obesity, adipokines and neuroinflammation. Neuropharmacology. 2015;96(Pt A):124-34.

43. Steculorum SM, Solas M, Brüning JC. The paradox of neuronal insulin action and resistance in the development of aging-associated diseases. Alzheimer's and Dementia. 2014;10(1 Suppl):S3-11.

44. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. 2011;472(7341):57–65.

45. Chen K, Zheng X, Feng M, Li D, Zhang H. Gut microbiota-dependent metabolite Trimethylamine N-oxide contributes to cardiac dysfunction in western diet-induced obese mice. Front Physiol. 2017;8:139.

46. Chen ML, Zhu XH, Ran L, Lang HD, Yi L, Mi MT. Trimethylamine-N-oxide induces vascular inflammation by activating the NLRP3 inflammasome through the SIRT3-SOD2-mtROS signaling pathway. J Am Heart Assoc. 2017;6(9):e006347.

47. Rohrmann S, Linseisen J, Allenspach M, Von Eckardstein A, Müller D. Plasma concentrations of trimethylamine- n-oxide are directly associated with dairy food consumption and low-grade inflammation in a german adult population. J Nutr. 2016;146(2):283-9.

48. Sun X, Jiao X, Ma Y, Liu Y, Zhang L, He Y, et al. Trimethylamine N-oxide induces inflammation and endothelial dysfunction in human umbilical vein endothelial cells via activating ROS-TXNIP-NLRP3 inflammasome. Biochem Biophys Res Commun. 2016;481(1–2):63–70.

49. Yue C, Yang X, Li J, Chen X, Zhao X, Chen Y, et al. Trimethylamine N-oxide prime NLRP3 inflammasome via inhibiting ATG16L1-induced autophagy in colonic epithelial cells. Biochem Biophys Res Commun. 2017;490(2):541-551.

50. Janeiro MH, Ramírez MJ, Milagro FI, Martínez JA, Solas M. Implication of trimethylamine n-oxide (TMAO) in disease: Potential biomarker or new therapeutic target. Nutrients. 2018;10(10):1398.

51. Fennema D, Phillips IR, Shephard EA. Trimethylamine and trimethylamine N-oxide, a Flavin-Containing Monooxygenase 3 (FMO3)-mediated host-microbiome metabolic axis implicated in health and disease. Drug Metabolism and Disposition. 2016;44(11):1839-1850.

52. Chhibber-Goel J, Gaur A, Singhal V, Parakh N, Bhargava B, Sharma A. The complex metabolism of trimethylamine in humans: Endogenous and exogenous sources. Expert Reviews in Molecular Medicine. 2016;18:e8.

53. Chen Y, Patel NA, Crombie A, Scrivens JH, Murrell JC. Bacterial flavin-containing monooxygenase is trimethylamine monooxygenase. Proc Natl Acad Sci U S A. 2011;108(43):17791-6.

54. Zhu Y, Jameson E, Crosatti M, Schäfer H, Rajakumar K, Bugg TDH, et al. Carnitine metabolism to trimethylamine by an unusual Rieske-type oxygenase from human microbiota. Proc Natl Acad Sci U S A. 2014;111(11):4268-73.

55. Zeisel SH, Warrier M. Trimethylamine N-Oxide, the Microbiome, and Heart and Kidney Disease. Annual Review of Nutrition. 2017;37:157-181.

56. Al-Waiz M, Mikov M, Mitchell SC, Smith RL. The exogenous origin of trimethylamine in the mouse. Metabolism. 1992;41(2):135-6.

57. Romano KA, Vivas EI, Amador-Noguez D, Rey FE. Intestinal microbiota composition modulates choline bioavailability from diet and accumulation of the proatherogenic metabolite trimethylamine-N-oxide. MBio. 2015;6(2):e02481.

58. Tang WHW, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, et al. Intestinal Microbial Metabolism of Phosphatidylcholine and Cardiovascular Risk. N Engl J Med. 2013;368:1575-1584.

59. Falony G, Vieira-Silva S, Raes J. Microbiology Meets Big Data: The Case of Gut Microbiota-Derived Trimethylamine. Annual Review of Microbiology. 2015;69:305-21.

60. Cho CE, Taesuwan S, Malysheva O V., Bender E, Tulchinsky NF, Yan J, et al. Trimethylamine-N-oxide (TMAO) response to animal source foods varies among healthy young men and is influenced by their gut microbiota composition: A randomized controlled trial. Mol Nutr Food Res. 2017;61(1).

61. Hui DY. Intestinal phospholipid and lysophospholipid metabolism in cardiometabolic disease. Current Opinion in Lipidology. 2016;27(5):507-12.

62. Taesuwan S, Cho CE, Malysheva O V., Bender E, King JH, Yan J, et al. The metabolic fate of isotopically labeled trimethylamine-N-oxide (TMAO) in humans. J Nutr Biochem. 2017;45:77-82.

63. Veeravalli S, Karu K, Phillips IR, Shephard EA. A highly sensitive liquid chromatography electrospray ionization mass spectrometry method for quantification of TMA, TMAO and creatinine in mouse urine. MethodsX. 2017;4:310-319.

64. Wahlang B, Barney J, Thompson B, Wang C, Hamad OM, Hoffman JB, et al. PCB126 exposure increases risk for peripheral vascular diseases in a liver injury mouse model. Toxicol Sci. 2017;160(2):256–67.

65. Zhang C, Yin A, Li H, Wang R, Wu G, Shen J, et al. Dietary Modulation of Gut Microbiota Contributes to Alleviation of Both Genetic and Simple Obesity in Children. EBioMedicine. 2015;2(8):968-84.

66. Wekell JC, Barnett H. New Method for Analysis of Trimethylamine Oxide Using Ferrous Sulfate and EDTA. J Food Sci. 1991;56(1):132-135.

67. Laxson CJ, Condon NE, Drazen JC, Yancey PH. Decreasing urea: Trimethylamine N-oxide ratios with depth in chondrichthyes: A physiological depth limit? Physiol Biochem Zool. 2011;84(5):494-505.

68. Zerbst-Boroffka I, Kamaltynow RM, Harjes S, Kinne-Saffran E, Gross J. TMAO and other organic osmolytes in the muscles of amphipods (Crustacea) from shallow and deep water of Lake Baikal. Comp Biochem Physiol - A Mol Integr Physiol. 2005;142(1):58-64.

69. Rio D Del, Zimetti F, Caffarra P, Tassotti M, Bernini F, Brighenti F, et al. The gut microbial metabolite trimethylamine-N-oxide is present in human cerebrospinal fluid. Nutrients. 2017;9(10):2–5.

70. Velasquez MT, Ramezani A, Manal A, Raj DS. Trimethylamine N-oxide: The good, the bad and the unknown. Toxins. 2016;8(11):326.

71. Li T, Chen Y, Gua C, Li X. Elevated circulating trimethylamine N-oxide levels contribute to endothelial dysfunction in aged rats through vascular inflammation and oxidative stress. Front Physiol. 2017;8:350.

72. Wang Z, Levison BS, Hazen JE, Donahue L, Li XM, Hazen SL. Measurement of trimethylamine-N-oxide by stable isotope dilution liquid chromatography tandem mass spectrometry. Anal Biochem. 2014;455:35-40.

73. Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota metabolism of l-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med. 2013;19(5):576-85.

74. Boutagy NE, Neilson AP, Osterberg KL, Smithson AT, Englund TR, Davy BM, et al. Short-term high-fat diet increases postprandial trimethylamine-N-oxide in humans. Nutr Res. 2015;35(10):858–64.

75. Gao X, Xu J, Jiang C, Zhang Y, Xue Y, Li Z, et al. Fish oil ameliorates trimethylamine N-oxide-exacerbated glucose intolerance in high-fat diet-fed mice. Food Funct. 2015;6(4):1117–25.

76. Sun G, Yin Z, Liu N, Bian X, Yu R, Su X, et al. Gut microbial metabolite TMAO contributes to renal dysfunction in a mouse model of diet-induced obesity. Biochem Biophys Res Commun. 2017;493(2):964–70.

77. Sanguinetti E, Collado MC, Marrachelli VG, Monleon D, Selma-Royo M, Pardo-Tendero MM, et al. Microbiome-metabolome signatures in mice genetically prone to develop dementia, fed a normal or fatty diet. Sci Rep. 2018;8(1):1–13.

78. Neilson A, Ponder M, Stewart A, Tmao KT, Tma T. The Impact of Dietary Fat and Phosphatidylcholine on Increased By Reem Ajlan Thesis submitted to the Faculty of Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of : Master of Life Science in Fo. 2017.

79. Washburn RL, Cox JE, Muhlestein JB, May HT, Carlquist JF, Le VT, et al. Pilot study of novel intermittent fasting effects on metabolomic and trimethylamine N-oxide changes during 24-hour water-only fasting in the FEELGOOD trial. Nutrients. 2019;11(2).

80. Rasmussen LG, Winning H, Savorani F, Toft H, Larsen TM, Dragsted LO, et al. Assessment of the effect of high or low protein diet on the human urine metabolome as measured by NMR. Nutrients. 2012;4(2):112-31.

81. Ufnal M, Zadlo A, Ostaszewski R. TMAO: A small molecule of great expectations. Nutrition. 2015;31(11-12):1317-23.

82. Mafra D, Borges NA, Cardozo LFM de F, Anjos JS, Black AP, Moraes C, et al. Red meat intake in chronic kidney disease patients: Two sides of the coin. Nutrition. 2018;46:26-32.

83. Bergeron N, Williams PT, Lamendella R, Faghihnia N, Grube A, Li X, et al. Diets high in resistant starch increase plasma levels of trimethylamine-N-oxide, a gut microbiome metabolite associated with CVD risk. Br J Nutr. 2016;116(12):2020-2029.

84. Wang X, Li X, Dong Y. Vitamin D Decreases Plasma Trimethylamine-N-oxide Level in Mice by Regulating Gut Microbiota. Biomed Res Int. 2020;2020:1-11.

85. Hernández-Alonso P, Cañueto D, Giardina S, Salas-Salvadó J, Cañellas N, Correig X, et al. Effect of pistachio consumption on the modulation of urinary gut microbiota-related metabolites in prediabetic subjects. J Nutr Biochem. 2017;45:48-53.

86. Du S, Sun S, Liu L, Zhang Q, Guo F, Li C, et al. Effects of Histidine Supplementation on Global Serum and Urine 1H NMR-based Metabolomics and Serum Amino Acid Profiles in Obese Women from a Randomized Controlled Study. J Proteome Res. 2017;16(6):2221-2230.

87. Obeid R, Awwad HM, Rabagny Y, Graeber S, Herrmann W, Geisel J. Plasma trimethylamine N-oxide concentration is associated with choline, phospholipids, and methyl metabolism. Am J Clin Nutr. 2016;103(3):703-11.

88. Mueller DM, Allenspach M, Othman A, Saely CH, Muendlein A, Vonbank A, et al. Plasma levels of trimethylamine-N-oxide are confounded by impaired kidney function and poor metabolic control. Atherosclerosis. 2015;243(2):638-44.

89. Stubbs JR, House JA, Ocque AJ, Zhang S, Johnson C, Kimber C, et al. Serum Trimethylamine-N-Oxide is Elevated in CKD and Correlates with Coronary Atherosclerosis Burden. J Am Soc Nephrol. 2016;27(1):305-13.

90. Missailidis C, Hällqvist J, Qureshi AR, Barany P, Heimbürger O, Lindholm B, et al. Serum trimethylamine-N-Oxide is strongly related to renal function and predicts outcome in chronic kidney disease. PLoS One. 2016;11(1):e0141738.

91. Tang WH, Wang Z, Kennedy DJ, Wu Y, Buffa JA, Agatisa-Boyle B, et al. Gut microbiota-dependent trimethylamine N-oxide (TMAO) pathway contributes to both development of renal insufficiency and mortality risk in chronic kidney disease. Circ Res. 2014;116(3):448-55.

92. Al-Ani B, Fitzpatrick M, Al-Nuaimi H, Coughlan AM, Hickey FB, Pusey CD, et al. Changes in urinary metabolomic profile during relapsing renal vasculitis. Sci Rep. 2016;6:38074.

93. Al-Obaide MAI, Singh R, Datta P, Rewers-Felkins KA, Salguero MV, Al-Obaidi I, Kottapalli KR, Vasylyeva TL. Gut Microbiota-Dependent Trimethylamine-N-oxide and Serum Biomarkers in Patients with T2DM and Advanced CKD. J Clin Med. 2017;6(9):86.

94. Wang M, Wang F, Wang Y, Ma X, Zhao M, Zhao C. Metabonomics study of the therapeutic mechanism of gynostemma pentaphyllum and atorvastatin for hyperlipidemia in rats. PLoS One. 2013;8(11):e78731.

95. Boutagy NE, Neilson AP, Osterberg KL, Smithson AT, Englund TR, Davy BM, et al. Probiotic supplementation and trimethylamine-N-oxide production following a high-fat diet. Obesity. 2015;23(12):2357-63.

96. Gao X, Xu J, Jiang C, Zhang Y, Xue Y, Li Z, et al. Fish oil ameliorates trimethylamine N-oxide-exacerbated glucose intolerance in high-fat diet-fed mice. Food Funct. 2015;6(4):1117-25.

97. Quigley EMM. Microbiota-Brain-Gut Axis and Neurodegenerative Diseases. Curr Neurol Neurosci Rep. 2017;17(12):94.

98. Bermon S, Petriz B, Kajeniene A, Prestes J, Castell L, Franco OL. The microbiota: An exercise immunology perspective. Exercise Immunology Review. 2015;21:70-9.

99. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59-65.

100. Vogt NM, Romano KA, Darst BF, Engelman CD, Johnson SC, Carlsson CM, et al. The gut microbiota-derived metabolite trimethylamine N-oxide is elevated in Alzheimer's disease. Alzheimer's Res Ther. 2018;10(1):1–8.

101. Enko D, Zelzer S, Niedrist T, Holasek S, Baranyi A, Schnedl WJ, et al. Assessment of trimethylamine-n-oxide at the blood-cerebrospinal fluid barrier: Results from 290 lumbar punctures. EXCLI J. 2020;19:1275–81.

102. Sankowski B, Księżarczyk K, Raćkowska E, Szlufik S, Koziorowski D, Giebułtowicz J. Higher cerebrospinal fluid to plasma ratio of p-cresol sulfate and indoxyl sulfate in patients with Parkinson's disease. Clin Chim Acta. 2020;501:165–73.

103. Zhang J, Cashman JR. Quantitative analysis of FMO gene mRNA levels in human tissues. Drug Metab Dispos. 2006;34(1):19–26.

104. Cashman JR, Zhang J. Human flavin-containing monooxygenases. Annual Review of Pharmacology and Toxicology. 2006;46:65-100.

105. Vernetti L, Gough A, Baetz N, Blutt S, Broughman JR, Brown JA, et al. Functional Coupling of Human Microphysiology Systems: Intestine, Liver, Kidney Proximal Tubule, Blood-Brain Barrier and Skeletal Muscle. Sci Rep. 2017;7:42296.

106. Brunt VE, LaRocca TJ, Bazzoni AE, Sapinsley ZJ, Miyamoto-Ditmon J, Gioscia-Ryan RA, et al. The gut microbiome–derived metabolite trimethylamine N-oxide modulates neuroinflammation and cognitive function with aging. GeroScience. 2020;43(1):377-394.

107. Subramaniam S, Fletcher C. Trimethylamine N-oxide: breathe new life. Br J Pharmacol. 2018;175(8):1344–53.

108. Boini KM, Hussain T, Li PL, Koka SS. Trimethylamine-N-Oxide Instigates NLRP3 Inflammasome Activation and Endothelial Dysfunction. Cell Physiol Biochem. 2017;44(1):152-162.

109. Zou Q, Bennion BJ, Daggett V, Murphy KP. The molecular mechanism of stabilization of proteins by TMAO and its ability to counteract the effects of urea. J Am Chem Soc. 2002;124(7):1192-202.

110. Tatzelt J, Prusiner SB, Welch WJ. Chemical chaperones interfere with the formation of scrapie prion protein. EMBO J. 1996;15(23):6363–73.

111. Yoshida H, Yoshizawa T, Shibasaki F, Shoji S, Kanazawa I. Chemical chaperones reduce aggregate formation and cell death caused by the truncated

Machado-Joseph disease gene product with an expanded polyglutamine stretch. Neurobiol Dis. 2002;10(2):88–99.

112. Getter T, Zaks I, Barhum Y, Ben-Zur T, Böselt S, Gregoire S, et al. A chemical chaperone-based drug candidate is effective in a mouse model of amyotrophic lateral sclerosis (ALS). ChemMedChem. 2015;10(5):850–61.

113. Jamal S, Kumari A, Singh A, Goyal S, Grover A. Conformational ensembles of α-synuclein derived peptide with different osmolytes from temperature replica exchange sampling. Front Neurosci. 2017;11:1–11.

114. Uversky VN, Li J, Fink AL. Trimethylamine-N-oxide-induced folding of alpha-synuclein. FEBS Lett. 2001;509(1):31-5.

115. Xu R, Wang QQ. Towards understanding brain-gut-microbiome connections in Alzheimer's disease. BMC Syst Biol. 2016;10 Suppl 3(Suppl 3):63.

116. Zhuang Z, Gao M, Yang R, Liu Z, Cao W, Huang T. Causal relationships between gut metabolites and Alzheimer's disease: a bidirectional Mendelian randomization study. Neurobiol Aging. 2020;258:2–5.

117. Tseng HC, Graves DJ. Natural methylamine osmolytes, trimethylamine N-oxide and betaine, increase tau-induced polymerization of microtubules. Biochem Biophys Res Commun. 1998;250(3):726–30.

118. Smith MJ, Crowther RA, Goedert M. The natural osmolyte trimethylamine N-oxide (TMAO) restores the ability of mutant tau to promote microtubule assembly. FEBS Lett. 2000;484(3):265–70.

119. Tseng HC, Lu Q, Henderson E, Graves DJ. Phosphorylated tau can promote tubulin assembly. Proc Natl Acad Sci U S A. 1999;96(17):9503–8.

120. Yang DS, Yip CM, Huang THJ, Chakrabartty A, Fraser PE. Manipulating the amyloid-β aggregation pathway with chemical chaperones. J Biol Chem. 1999;274(46):32970-4.

121. Chung SJ, Rim JH, Ji D, Lee S, Yoo HS, Jung JH, et al. Gut microbiota-derived metabolite trimethylamine N-oxide as a biomarker in early Parkinson's disease. Nutrition. 2021;83:111090.

122. Ferreon ACM, Moosa MM, Gambin Y, Deniz AA. Counteracting chemical chaperone effects on the single-molecule α-synuclein structural landscape. Proc Natl Acad Sci U S A. 2012;109(44):17826–31.

123. Pistollato F, Cano SS, Elio I, Vergara MM, Giampieri F, Battino M. Role of gut microbiota and nutrients in amyloid formation and pathogenesis of Alzheimer disease. Nutr Rev. 2016;74(10):624–34.

124. Wang S, Huang XF, Zhang P, Newell KA, Wang H, Zheng K, et al. Dietary teasaponin ameliorates alteration of gut microbiota and cognitive decline in diet-induced obese mice. Sci Rep. 2017;7(1):12203.

125. Brandscheid C, Schuck F, Reinhardt S, Schäfer KH, Pietrzik CU, Grimm M, et al. Altered Gut Microbiome Composition and Tryptic Activity of the 5xFAD Alzheimer's Mouse Model. J Alzheimer's Dis. 2017;56(2):775-788.

126. Parashar A, Udayabanu M. Gut microbiota: Implications in Parkinson's disease. Parkinsonism and Related Disorders. 2017;38:1-7.

127. van Praag H. Lifestyle Factors and Alzheimer's Disease. Brain Plast. 2018;4(1):1-2.

128. Grant WB. Trends in diet and Alzheimer's disease during the nutrition transition in Japan and developing countries. J Alzheimer's Dis. 2014;38(3):611-20.

129. Nagpal R, Mainali R, Ahmadi S, Wang S, Singh R, Kavanagh K, et al. Gut microbiome and aging: Physiological and mechanistic insights. Nutrition and Healthy Aging. 2018;4(4):267-285.

130. Salazar N, Valdés-Varela L, González S, Gueimonde M, de los Reyes-Gavilán CG. Nutrition and the gut microbiome in the elderly. Gut Microbes. 2017;8(2):82-97.

131. Zapata HJ, Quagliarello VJ. The microbiota and microbiome in aging: Potential implications in health and age-related diseases. J Am Geriatr Soc. 2015;63(4):776–81.

132. O'Toole PW, Jeffery IB. Gut microbiota and aging. Science. 2015;350(6265):1214–5.

133. Cenit MC, Sanz Y, Codoñer-Franch P. Influence of gut microbiota on neuropsychiatric disorders. World J Gastroenterol. 2017;23(30):5486–98.

134. Gavini F, Cayuela C, Antoine JM, Lecoq C, Lefebvre B, Membré JM, et al. Differences in the distribution of bifidobacterial and enterobacterial species in human faecal microflora of three different (children, adults, elderly) age groups. Microb Ecol Health Dis. 2001;13:40-45.

135. Hopkins MJ, Macfarlane GT. Changes in predominant bacterial populations in human faeces with age and with Clostridium difficile infection. J Med Microbiol. 2002;51(5):448-454.

136. He F, Ouwehand AC, Isolauri E, Hosoda M, Benno Y, Salminen S. Differences in composition and mucosal adhesion of bifidobacteria isolated from healthy adults and healthy seniors. Curr Microbiol. 2001;43(5):351-4.

137. Bartosch S, Fite A, Macfarlane GT, McMurdo MET. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly

patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl Environ Microbiol. 2004;70(6):3575-81.

138. Layton A, McKay L, Williams D, Garrett V, Gentry R, Sayler G. Development of Bacteroides 16S rRNA gene taqman-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Appl Environ Microbiol. 2006;72(6):4214-24.

139. Janeiro MH, Ramírez MJ, Solas M. Dysbiosis and Alzheimer's Disease: Cause or Treatment Opportunity? Cell Mol Neurobiol. 2021.

140. Wang Z, Levison BS, Hazen JE, Donahue L, Li X, Hazen SL. Measurement of TMAO by stable isotope dilution LC tandem mass spec. 2015;35–40.

141. Gao X, Sun G, Randell E, Tian Y, Zhou H. Systematic investigation of the relationships of trimethylamine: N -oxide and l-carnitine with obesity in both humans and rodents. Food Funct. 2020;11(9):7707–16.

142. Takeda T, Hosokawa M, Takeshita S, Irino M, Higuchi K, Matsushita T, et al. A new murine model of accelerated senescence. Mech Ageing Dev. 1981;17(2):183-94.

143. Flood JF, E. Morley J. Learning and memory in the SAMP8 mouse. Neurosci Biobehav Rev. 1998;22(1):1-20.

144. Takeda T. Senescence-accelerated mouse (SAM): A biogerontological resource in aging research. Neurobiol Aging. 1999;20(2):105-10.

145. Pallàs M, Camins A, Smith MA, Perry G, Lee HG, Casadesus G. From aging to Alzheimer's disease: Unveiling "The switch" with the senescence-accelerated mouse model (SAMP8). Journal of Alzheimer's Disease. 2008;15(4):615-24.

146. Pallàs M. Senescence-Accelerated Mice P8: A Tool to Study Brain Aging and Alzheimer's Disease in a Mouse Model. ISRN Cell Biol. 2012;2012.

147. Morley JE, Armbrecht HJ, Farr SA, Kumar VB. The senescence accelerated mouse (SAMP8) as a model for oxidative stress and Alzheimer's disease. Biochim Biophys Acta - Mol Basis Dis. 2012;1822(5):650-6.

148. Bayram B, Ozcelik B, Grimm S, Roeder T, Schrader C, Ernst IMA, et al. A diet rich in olive oil phenolics reduces oxidative stress in the heart of SAMP8 mice by induction of Nrf2-dependent gene expression. Rejuvenation Res. 2012;15(1):71-81.

149. Del Valle J, Duran-Vilaregut J, Manich G, Camins A, Pallàs M, Vilaplana J, et al. Time-course of blood-brain barrier disruption in senescence-accelerated mouse prone 8 (SAMP8) mice. Int J Dev Neurosci. 2009;27(1):47-52.

150. Cuesta S, Kireev R, Forman K, García C, Escames G, Ariznavarreta C, et al. Melatonin improves inflammation processes in liver of senescence-accelerated prone male mice (SAMP8). Exp Gerontol. 2010;45(12):950-6.

151. Porquet D, Casadesús G, Bayod S, Vicente A, Canudas AM, Vilaplana J, et al. Dietary resveratrol prevents Alzheimer's markers and increases life span in SAMP8. Age (Omaha). 2013;35(5):1851-65.

152. Manich G, Mercader C, Del Valle J, Duran-Vilaregut J, Camins A, Pallàs M, et al. Characterization of amyloid-β granules in the hippocampus of SAMP8 mice. J Alzheimer's Dis. 2011;25(3):535-46.

153. Del Valle J, Duran-Vilaregut J, Manich G, Casadesús G, Smith MA, Camins A, et al. Early amyloid accumulation in the hippocampus of SAMP8 mice. J Alzheimer's Dis. 2010;19(4):1303-15.

154. Zhang X, Li G, Guo L, Nie K, Jia Y, Zhao L, et al. Age-related alteration in cerebral blood flow and energy failure is correlated with cognitive impairment in the senescence-accelerated prone mouse strain 8 (SAMP8). Neurol Sci. 2013;34(11):1917–24.

155. Ke Y, Li D, Zhao M, Liu C, Liu J, Zeng A, et al. Gut flora-dependent metabolite Trimethylamine-N-oxide accelerates endothelial cell senescence and vascular aging through oxidative stress. Free Radic Biol Med. 2018;116:88–100.

156. Saco T, Parthasarathy PT, Cho Y, Lockey RF, Kolliputi N. Inflammasome: A new trigger of Alzheimer's disease. Frontiers in Aging Neuroscience. 2014;6:80.

157. Kelly JR, Kennedy PJ, Cryan JF, Dinan TG, Clarke G, Hyland NP. Breaking down the barriers: The gut microbiome, intestinal permeability and stress-related psychiatric disorders. Frontiers in Cellular Neuroscience. 2015;9:392.

158. Luca M, Luca A, Calandra C. The Role of Oxidative Damage in the Pathogenesis and Progression of Alzheimer's Disease and Vascular Dementia. Oxidative Medicine and Cellular Longevity. 2015;2015:504678.

159. Minter MR, Zhang C, Leone V, Ringus DL, Zhang X, Oyler-Castrillo P, et al. Antibiotic-induced perturbations in gut microbial diversity influences neuro-inflammation and amyloidosis in a murine model of Alzheimer's disease. Sci Rep. 2016;6:30028.

160. Cattaneo A, Cattane N, Galluzzi S, Provasi S, Lopizzo N, Festari C, et al. Association of brain amyloidosis with pro-inflammatory gut bacterial taxa and peripheral inflammation markers in cognitively impaired elderly. Neurobiol Aging. 2017;49:60-68.

161. Zhao Y, Jaber V, Lukiw WJ. Secretory products of the human GI tract microbiome and their potential impact on Alzheimer's disease (AD): Detection of lipopolysaccharide (LPS) in AD hippocampus. Front Cell Infect Microbiol. 2017;7:318.

162. Hauss-Wegrzyniak B, Wenk GL. Beta-amyloid deposition in the brains of rats chronically infused with thiorphan or lipopolysaccharide: The role of ascorbic acid in the vehicle. Neurosci Lett. 2002;322(2):75-8.

163. Zhao Y, Lukiw WJ. Bacteroidetes Neurotoxins and Inflammatory Neurodegeneration. Molecular Neurobiology. 2018;55(12):9100-9107.

164. Bhattacharjee S, Zhao Y, Dua P, Rogaev EI, Lukiw WJ. MicroRNA-34α-mediated down-regulation of the microglial-enriched triggering receptor and phagocytosis-sensor TREM2 in age-related macular degeneration. PLoS One. 2016;11(3):e0150211.

165. Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, et al. Structural and functional features of central nervous system lymphatic vessels. Nature. 2015;523(7560):337-41.

166. Saksida T, Koprivica I, Vujičić M, Stošić-Grujičić S, Perović M, Kanazir S, et al. Impaired IL-17 Production in Gut-Residing Immune Cells of 5xFAD Mice with Alzheimer's Disease Pathology. J Alzheimer's Dis. 2017;61(2):619-630.

167. Koutrolos M, Berer K, Kawakami N, Wekerle H, Krishnamoorthy G. Treg cells mediate recovery from EAE by controlling effector T cell proliferation and motility in the CNS. Acta Neuropathologica Communications. 2014;2:163.

168. Wekerle H. The gut-brain connection: Triggering of brain autoimmune disease by commensal gut bacteria. Rheumatol (United Kingdom). 2016;55(suppl 2):ii68-ii75.

169. Bonfili L, Cecarini V, Berardi S, Scarpona S, Suchodolski JS, Nasuti C, et al. Microbiota modulation counteracts Alzheimer's disease progression influencing neuronal proteolysis and gut hormones plasma levels. Sci Rep. 2017;7(1):2426.

170. Chen D, Yang X, Yang J, Lai G, Yong T, Tang X, et al. Prebiotic effect of Fructooligosaccharides from Morinda officinalis on Alzheimer's disease in rodent models by targeting the microbiota-gut-brain axis. Front Aging Neurosci. 2017;9:403.

171. Erny D, De Angelis ALH, Jaitin D, Wieghofer P, Staszewski O, David E, et al. Host microbiota constantly control maturation and function of microglia in the CNS. Nat Neurosci. 2015;18(7):965-77.

172. Rothhammer V, Mascanfroni ID, Bunse L, Takenaka MC, Kenison JE, Mayo L, et al. Type i interferons and microbial metabolites of tryptophan modulate astrocyte activity and central nervous system inflammation via the aryl hydrocarbon receptor. Nat Med. 2016;22(6):586-97.

173. Zhang R, Miller RG, Gascon R, Champion S, Katz J, Lancero M, et al. Circulating endotoxin and systemic immune activation in sporadic amyotrophic lateral sclerosis (sALS). J Neuroimmunol. 2009;206(1-2):121-4.

174. Calsolaro V, Edison P. Neuroinflammation in Alzheimer's disease: Current evidence and future directions. Alzheimer's and Dementia. 2016;12(6):719-32.

175. Spielman LJ, Gibson DL, Klegeris A. Unhealthy gut, unhealthy brain: The role of the intestinal microbiota in neurodegenerative diseases. Neurochemistry International. 2018;120:149-163.

176. Zhao Y, Cong L, Jaber V, Lukiw WJ. Microbiome-derived lipopolysaccharide enriched in the perinuclear region of Alzheimer's disease brain. Front Immunol. 2017;8:1064.

177. Martin FPJ, Wang Y, Sprenger N, Yap IKS, Lundstedt T, Lek P, et al. Probiotic modulation of symbiotic gut microbial-host metabolic interactions in a humanized microbiome mouse model. Mol Syst Biol. 2008;4:157.

178. Brugère JF, Borrel G, Gaci N, Tottey W, O'Toole PW, Malpuech-Brugère C. Archaebiotics: Proposed therapeutic use of archaea to prevent trimethylaminuria and cardiovascular disease. Gut Microbes. 2013;5(1):5-10.

179. Dridi B, Fardeau ML, Ollivier B, Raoult D, Drancourt M. Methanomassiliicoccus luminyensis gen. nov., sp. nov., a methanogenic archaeon isolated from human faeces. Int J Syst Evol Microbiol. 2012;62(Pt 8):1902-1907.

180. Mendelsohn AR, Larrick JW. Dietary modification of the microbiome affects risk for cardiovascular disease. Rejuvenation Research. 2013;16(3):241-4.

181. Nagatomo Y, Tang WHW. Intersections between Microbiome and Heart Failure: Revisiting the Gut Hypothesis. Journal of Cardiac Failure. 2015;21(12):973-80.

182. Miao J, Ling A V., Manthena P V., Gearing ME, Graham MJ, Crooke RM, et al. Flavin-containing monooxygenase 3 as a potential player in diabetes-associated atherosclerosis. Nat Commun. 2015;6:6498.

183. Warrier M, Shih DM, Burrows AC, Ferguson D, Gromovsky AD, Brown AL, et al. The TMAO-Generating Enzyme Flavin Monooxygenase 3 Is a Central Regulator of Cholesterol Balance. Cell Rep. 2015;10(3):326-338.

184. Shih DM, Wang Z, Lee R, Meng Y, Che N, Charugundla S, et al. Flavin containing monooxygenase 3 exerts broad effects on glucose and lipid metabolism and atherosclerosis. J Lipid Res. 2015;56(1):22-37.

185. Sun B, Wang X, Cao R, Zhang Q, Liu Q, Xu M, et al. NMR-based metabonomics study on the effect of Gancao in the attenuation of toxicity in rats induced by Fuzi. J Ethnopharmacol. 2016;193:617-626.

186. Chen ML, Yi L, Zhang Y, Zhou X, Ran L, Yang J, et al. Resveratrol attenuates trimethylamine-N-oxide (TMAO)-induced atherosclerosis by regulating TMAO

synthesis and bile acid metabolism via remodeling of the gut microbiota. MBio. 2016;7(2):e02210-15.

187. Konop M, Radkowski M, Grochowska M, Perlejewski K, Samborowska E, Ufnal M. Enalapril decreases rat plasma concentration of TMAO, gut bacteria-derived cardiovascular marker. Biomarkers. 2018;23(4):380-385.

188. Dambrova M, Skapare-makarova E, Konrade I, Pugovics O, Grinberga S, Tirzite D, et al. Meldonium decreases the diet-increased plasma levels of trimethylamine n-oxide, a metabolite associated with atherosclerosis. J Clin Pharmacol. 2013;53(10):1095–8.

189. Kuka J, Liepinsh E, Makrecka-Kuka M, Liepins J, Cirule H, Gustina D, et al. Suppression of intestinal microbiota-dependent production of pro-atherogenic trimethylamine N-oxide by shifting L-carnitine microbial degradation. Life Sci. 2014;117(2):84–92.

190. Spector R. New insight into the dietary cause of atherosclerosis: Implications for Pharmacology. Journal of Pharmacology and Experimental Therapeutics. 2016;358(1):103-8. .

191. Wang Z, Roberts AB, Buffa JA, Levison BS, Zhu W, Org E, et al. Non-lethal inhibition of gut microbial trimethylamine production for the treatment of atherosclerosis assisted in gene cloning, protein purification, choline transport and TMA lyase activity assay HHS Public Access. Cell. 2015;163(7):1585–95.

192. Chen H, Li J, Li N, Liu H, Tang J. Increased circulating trimethylamine N-oxide plays a contributory role in the development of endothelial dysfunction and hypertension in the RUPP rat model of preeclampsia. Hypertens Pregnancy. 2019;38(2):96–104.

193. Gao Q, Wang Y, Wang X, Fu S, Zhang X, Wang RT, et al. Decreased levels of circulating trimethylamine N-oxide alleviate cognitive and pathological deterioration in transgenic mice: A potential therapeutic approach for Alzheimer's disease. Aging (Albany NY). 2019;11(19):8642–63.

194. Chen L, Jin Y, Wang N, Yuan M, Lin T, Lu W, et al. Trimethylamine N-oxide impairs perfusion recovery after hindlimb ischemia. Biochem Biophys Res Commun. 2020;530(1):95-99.

195. Wang G, Kong B, Shuai W, Fu H, Jiang X, Huang H. 3,3-Dimethyl-1-butanol attenuates cardiac remodeling in pressure-overload-induced heart failure mice. J Nutr Biochem. 2020;78:108341.

196. Shuai W, Wen J, Li X, Wang D, Li Y, Xiang J. High-Choline Diet Exacerbates Cardiac Dysfunction, Fibrosis, and Inflammation in a Mouse Model of Heart Failure With Preserved Ejection Fraction. J Card Fail. 2020;26(8):694-702.

197. Brunt VE, Gioscia-Ryan RA, Casso AG, Vandongen NS, Ziemba BP, Sapinsley ZJ, et al. Trimethylamine-N-Oxide Promotes Age-Related Vascular Oxidative Stress and Endothelial Dysfunction in Mice and Healthy Humans. Hypertension. 2020;76(1):101–12.

198. Hsu CN, Chang-Chien GP, Lin S, Hou CY, Tain YL. Targeting on Gut Microbial Metabolite Trimethylamine-N-Oxide and Short-Chain Fatty Acid to Prevent Maternal High-Fructose-Diet-Induced Developmental Programming of Hypertension in Adult Male Offspring. Mol Nutr Food Res. 2019;63(18):1–10.

199. Li X, Sun Y, Zhang X, Wang J. Reductions in gut microbiota-derived metabolite trimethylamine N-oxide in the circulation may ameliorate myocardial infarction-induced heart failure in rats, possibly by inhibiting interleukin-8 secretion. Mol Med Rep. 2019;20(1):779-786.

200. Li T, Gua C, Wu B, Chen Y. Increased circulating trimethylamine N-oxide contributes to endothelial dysfunction in a rat model of chronic kidney disease. Biochem Biophys Res Commun. 2018;495(2):2071-2077.

201. Yang W, Zhang S, Zhu J, Jiang H, Jia D, Ou T, et al. Gut microbe-derived metabolite trimethylamine N-oxide accelerates fibroblast-myofibroblast differentiation and induces cardiac fibrosis. J Mol Cell Cardiol. 2019;134:119-130.

202. Gorelick PB. Role of inflammation in cognitive impairment: Results of observational epidemiological studies and clinical trials. Annals of the New York Academy of Sciences. 2010;1207:155-62.

203. Spencer SJ. Perinatal nutrition programs neuroimmune function long-term: Mechanisms and implications. Front Neurosci. 2013;7:144.

204. Hanzel CE, Pichet-Binette A, Pimentel LSB, Iulita MF, Allard S, Ducatenzeiler A, et al. Neuronal driven pre-plaque inflammation in a transgenic rat model of Alzheimer's disease. Neurobiol Aging. 2014;35(10):2249-62.

205. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, et al. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell. 2013;155(7):1451-63.

206. Bruce-Keller AJ, Salbaum JM, Luo M, Blanchard E, Taylor CM, Welsh DA, et al. Obese-type gut microbiota induce neurobehavioral changes in the absence of obesity. Biol Psychiatry. 2015;77(7):607-15.

207. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014;505(7484):559-63.

208. Noble EE, Hsu TM, Jones RB, Fodor AA, Goran MI, Kanoski SE. Early-life sugar consumption affects the rat microbiome independently of obesity. J Nutr. 2017;147(1):20-28.

209. Solas M, Aisa B, Mugueta MC, Del Río J, Tordera RM, Ramírez MJ. Interactions between age, stress and insulin on cognition: implications for Alzheimer's disease. Neuropsychopharmacology. 2010;35(8):1664–73.

210. Bell RD, Winkler EA, Sagare AP, Singh I, LaRue B, Deane R, et al. Pericytes Control Key Neurovascular Functions and Neuronal Phenotype in the Adult Brain and during Brain Aging. Neuron. 2010;68(3):409-427.

211. Bell RD, Winkler EA, Singh I, Sagare AP, Deane R, Wu Z, et al. Apolipoprotein e controls cerebrovascular integrity via cyclophilin A. Nature. 2012;485(7399):512-516.

212. Winkler EA, Sengillo JD, Bell RD, Wang J, Zlokovic B V. Blood-spinal cord barrier pericyte reductions contribute to increased capillary permeability. J Cereb Blood Flow Metab. 2012;32(10):1841-1852.

213. Winkler EA, Sengillo JD, Sullivan JS, Henkel JS, Appel SH, Zlokovic B V. Blood-spinal cord barrier breakdown and pericyte reductions in amyotrophic lateral sclerosis. Acta Neuropathol. 2013;125(1):111-120.

214. Weiss S, Xu Z, Amir A, Peddada S, Bittinger K, Gonzalez A, et al. Effects of library size variance, sparsity, and compositionality on the analysis of microbiome data. PeerJ Prepr. 2015;3:e1157v1.

215. Zhao X, Zeisel SH, Zhang S. Rapid LC-MRM-MS assay for simultaneous quantification of choline, betaine, trimethylamine, trimethylamine N-oxide, and creatinine in human plasma and urine. Electrophoresis. 2015;36(18):2207-2214.

216. Ballard C, Gauthier S, Corbett A, Brayne C, Aarsland D, Jones E. Alzheimer's disease. The Lancet. 2011;377(9770):1019-31.

217. Mokdad AH, Ford ES, Bowman BA, Dietz WH, Vinicor F, Bales VS, et al. Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. J Am Med Assoc. 2003;289(1):76-9.

218. Frisardi V, Solfrizzi V, Seripa D, Capurso C, Santamato A, Sancarlo D, et al. Metabolic-cognitive syndrome: A cross-talk between metabolic syndrome and Alzheimer's disease. Ageing Research Reviews. 2010;9(4):399-417.

219. Frölich L, Blum-Degen D, Bernstein HG, Engelsberger S, Humrich J, Laufer S, et al. Brain insulin and insulin receptors in aging and sporadic Alzheimer's disease. J Neural Transm. 1998;105(4-5):423-38.

220. de Fernandes MLLA, Saad MJA, Velloso LA. Effects of age on elements of insulin-signaling pathway in central nervous system of rats. Endocrine. 2001;;16(3):227-34.

221. De Felice FG. Alzheimer's disease and insulin resistance: Translating basic science into clinical applications. J Clin Invest. 2013;123(2):531-9.

222. Bruning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, et al. Role of brain insulin receptor in control of body weight and reproduction. Science. 2000;289(5487):2122-5.

223. Brown MW, Aggleton JP. Recognition memory: What are the roles of the perirhinal cortex and hippocampus? Nat Rev Neurosci. 2001;2(1):51-61.

224. Banting FG, Best CH. The internal secretion of the pancreas. J Lab Clin Med. 1922;7: 256-271.

225. Fernandez AM, Torres-Alemán I. The many faces of insulin-like peptide signalling in the brain. Nature Reviews Neuroscience. 2012;13(4):225-39.

226. Kushner JA. The role of aging upon β cell turnover. Journal of Clinical Investigation. 2013;123(3):990-5.

227. Zhao WQ, Chen H, Quon MJ, Alkon DL. Insulin and the insulin receptor in experimental models of learning and memory. Eur J Pharmacol. 2004;490(1-3):71-81.

228. Vogt MC, Brüning JC. CNS insulin signaling in the control of energy homeostasis and glucose metabolism - from embryo to old age. Trends in Endocrinology and Metabolism. 2013;24(2):76-84.

229. Thaler JP, Guyenet SJ, Dorfman MD, Wisse BE, Schwartz MW. Hypothalamic inflammation: Marker or mechanism of obesity pathogenesis? Diabetes. 2013;62(8):2629-34.

230. Velloso LA, Schwartz MW. Altered hypothalamic function in diet-induced obesity. International Journal of Obesity. 2011;35(12):1455-65.

231. Könner AC, Brüning JC. Toll-like receptors: Linking inflammation to metabolism. Trends in Endocrinology and Metabolism. 2011;22(1):16-23.

232. De Souza CT, Araujo EP, Bordin S, Ashimine R, Zollner RL, Boschero AC, et al. Consumption of a fat-rich diet activates a proinflammatory response and induces insulin resistance in the hypothalamus. Endocrinology. 2005;146(10):4192-9.

233. Posey KA, Clegg DJ, Printz RL, Byun J, Morton GJ, Vivekanandan-Giri A, et al. Hypothalamic proinflammatory lipid accumulation, inflammation, and insulin resistance in rats fed a high-fat diet. Am J Physiol - Endocrinol Metab. 2009;296(5):E1003-12.

234. Zhang X, Zhang G, Zhang H, Karin M, Bai H, Cai D. Hypothalamic IKKβ/NF-κB and ER Stress Link Overnutrition to Energy Imbalance and Obesity. Cell. 2008;135(1):61-73.

235. Aguirre V, Uchida T, Yenush L, Davis R, White MF. The c-Jun NH2-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser307. J Biol Chem. 2000;275(12):9047-54.

236. Zhang G, Li J, Purkayastha S, Tang Y, Zhang H, Yin Y, et al. Hypothalamic programming of systemic ageing involving IKK-β, NF-κB and GnRH. Nature. 2013;497(7448):211-6.

237. Lee CK, Weindruch R, Prolla TA. Gene-expression profile of the ageing brain in mice. Nat Genet. 2000;25(3):294-7.

238. Cai Z, Qiao PF, Wan CQ, Cai M, Zhou NK, Li Q. Role of Blood-Brain Barrier in Alzheimer's Disease. Journal of Alzheimer's Disease. 2018;63(4):1223-1234.

239. Gregory JC, Buffa JA, Org E, Wang Z, Levison BS, Zhu W, et al. Transmission of atherosclerosis susceptibility with gut microbial transplantation. J Biol Chem. 2015;290(9):5647-60.

240. O'Connor A, Quizon PM, Albright JE, Lin FT, Bennett BJ. Responsiveness of cardiometabolic-related microbiota to diet is influenced by host genetics. Mamm Genome. 2014;25(11-12):583-99.

241. Carmody RN, Gerber GK, Luevano JM, Gatti DM, Somes L, Svenson KL, et al. Diet dominates host genotype in shaping the murine gut microbiota. Cell Host Microbe. 2015;17(1):72-84.

242. Leigh SJ, Morris MJ. Diet, inflammation and the gut microbiome: Mechanisms for obesity-associated cognitive impairment. Biochim Biophys Acta - Mol Basis Dis. 2020;1866(6):165767.

243. Studzinski CM, Li F, Bruce-Keller AJ, Fernandez-Kim SO, Zhang L, Weidner AM, et al. Effects of short-term Western diet on cerebral oxidative stress and diabetes related factors in APP x PS1 knock-in mice. J Neurochem. 2009;108(4):860-6.

244. Nam KN, Mounier A, Wolfe CM, Fitz NF, Carter AY, Castranio EL, et al. Effect of high fat diet on phenotype, brain transcriptome and lipidome in Alzheimer's model mice. Sci Rep. 2017;7(1):4307.

245. Sah SK, Lee C, Jang JH, Park GH. Effect of high-fat diet on cognitive impairment in triple-transgenic mice model of Alzheimer's disease. Biochem Biophys Res Commun. 2017;493(1):731-736.

246. Mohammadi A, Najar AG, Yaghoobi MM, Jahani Y, Vahabzadeh Z. Trimethylamine-N-Oxide Treatment Induces Changes in the ATP-Binding Cassette

Transporter A1 and Scavenger Receptor A1 in Murine Macrophage J774A.1 cells. Inflammation. 2016;39(1):393-404.

247. Schugar RC, Shih DM, Warrier M, Helsley RN, Burrows A, Ferguson D, et al. The TMAO-Producing Enzyme Flavin-Containing Monooxygenase 3 Regulates Obesity and the Beiging of White Adipose Tissue. Cell Rep. 2017;19(12):2451–61.

248. Geng J, Yang C, Wang B, Zhang X, Hu T, Gu Y, et al. Trimethylamine N-oxide promotes atherosclerosis via CD36-dependent MAPK/JNK pathway. Biomed Pharmacother. 2018;97:941–7.

249. Dumas ME, Rothwell AR, Hoyles L, Aranias T, Chilloux J, Calderari S, et al. Microbial-Host Co-metabolites Are Prodromal Markers Predicting Phenotypic Heterogeneity in Behavior, Obesity, and Impaired Glucose Tolerance. Cell Rep. 2017;20(1):136–48.

250. Mohammadi A, Vahabzadeh Z, Jamalzadeh S, Khalili T. Trimethylamine-N-oxide, as a risk factor for atherosclerosis, induces stress in J774A.1 murine macrophages. Adv Med Sci. 2018;63(1):57-63.

251. Zhang X, Li Y, Yang P, Liu X, Lu L, Chen Y, et al. Trimethylamine-N-oxide promotes vascular calcification through activation of NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) inflammasome and NF-κB (nuclear factor κb) signals. Arterioscler Thromb Vasc Biol. 2020;3:751–65.

252. Yang S, Li X, Yang F, Zhao R, Pan X, Liang J, et al. Gut microbiota-dependent marker TMAO in promoting cardiovascular disease: Inflammation mechanism, clinical prognostic, and potential as a therapeutic target. Front Pharmacol. 2019;10:1–14.

253. Dakkak W TA. Trimethylamine-N-Oxide Instigates NLRP3 Inflammasome Activation and Endothelial Dysfunction. Physiol Behav. 2017;176(5):139–48.

254. Janda E, Boi L, Carta AR. Microglial phagocytosis and its regulation: A therapeutic target in parkinson's disease? Front Mol Neurosci. 2018;11:1–8.

255. Zhou T, Huang Z, Zhu X, Sun X, Liu Y, Cheng B, et al. Alpha-1 antitrypsin attenuates M1 microglia-mediated neuroinflammation in retinal degeneration. Front Immunol. 2018;9:1202.

256. Yip PK, Bowes AL, Hall JCE, Burguillos MA, Richard Ip TH, Baskerville T, et al. Docosahexaenoic acid reduces microglia phagocytic activity via miR-124 and induces neuroprotection in rodent models of spinal cord contusion injury. Hum Mol Genet. 2019; o. Hum Mol Genet. 2019;28(14):2427-2448.

257. Kavanagh E, Burguillos MA, Carrillo-Jimenez A, Oliva-Martin MJ, Santiago M, Rodhe J, et al. Deletion of caspase-8 in mouse myeloid cells blocks microglia proinflammatory activation and confers protection in MPTP neurodegeneration model. Aging (Albany NY). 2015;7(9):673-89.

258. Green DR, Oguin TH, Martinez J. The clearance of dying cells: Table for two. Cell Death and Differentiation. 2016;23(6):915-26.

259. Li W. Eat-me signals: Keys to molecular phagocyte biology and “Appetite” control. J Cell Physiol. 2012;227(4):1291–7.

260. Takahashi K, Rochford CDP, Neumann H. Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. J Exp Med. 2005;201(4):647-57.

261. Siddiqui TA, Lively S, Schlichter LC. Complex molecular and functional outcomes of single versus sequential cytokine stimulation of rat microglia. J Neuroinflammation. 2016;13(1):66.

262. Fricker M, Oliva-Martín MJ, Brown GC. Primary phagocytosis of viable neurons by microglia activated with LPS or Aβ is dependent on calreticulin/LRP phagocytic signalling. J Neuroinflammation. 2012;9:196.

263. Khalili A, Nekooeian AA, Khosravi MB. Oleuropein improves glucose tolerance and lipid profile in rats with simultaneous renovascular hypertension and type 2 diabetes. J Asian Nat Prod Res. 2017;19(10):1011-1021.

264. Mount PF, Kemp BE, Power DA. Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation. Journal of Molecular and Cellular Cardiology. 2007;42(2):271-9.

265. Herrera MD, Mingorance C, Rodríguez-Rodríguez R, Alvarez de Sotomayor M. Endothelial dysfunction and aging: An update. Ageing Research Reviews. 2010;9(2):142-52.

266. Borges BC, Rorato R, Antunes-Rodrigues J, Elias LLK. Glial cell activity is maintained during prolonged inflammatory challenge in rats. Brazilian J Med Biol Res. 2012;45(8):784-91.

267. Eng LF, Ghirnikar RS. GFAP and Astrogliosis. In: Brain Pathology. 1994;4(3):229-37.