# UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE PSICOLOGÍA


# TESIS DOCTORAL

Papel de las apolipoproteinas y los componentes bacterianos en las alteraciones neuroconductuales inducidas por el consumo intensivo de alcohol

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MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

Leticia López Valencia

DIRIGIDA POR

Laura Orío Ortiz

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PAPEL DE LAS APOLIPOPROTEINAS Y LOS COMPONENTES BACTERIANOS EN LAS ALTERACIONES NEUROCONDUCTUALES INDUCIDAS POR EL CONSUMO INTENSIVO DE ALCOHOL

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# AGRADECIMIENTOS / ACKNOWLEDGEMENTS

Sin duda la defensa de esta Tesis Doctoral cierra una de la etapas más emocionantes e importantes de mi vida. No sólo ha sido el comienzo de mi carrera como investigadora y la confirmación de cuánto me apasiona la ciencia, sino que, en lo personal, he vivido numerosas experiencias que han marcado también mi paso por el Doctorado.

Mil millones de gracias a mi directora **Laura Orío,** por darme la oportunidad de haber podido despegar en este mundo que conocemos complejo. Tú me viste y escogiste, me has guiado durante el camino y has confiado en mi desde el principio. Gracias de corazón.

A mis compañeros durante estos años: **Javi, Víctor, Antonio, Berta, Eva Luna**. **Alicia** y **Marta**, cuánto me habéis enseñado y qué alegría me da siempre volver a veros. Un ratito con vosotras es alegría para el corazón. A mi **Sara Peñasco**, llegaste más tarde, pero te ganaste mi cariño y confianza desde el primer día. En lo profesional, qué gusto es trabajar contigo. Me has enseñado muchísimo en este tiempo. Sin duda eres una gran docente e investigadora. En lo personal, gracias por estar siempre dispuesta a echarme una mano sin esperar nada a cambio y por alegrarte de mis logros como si fueran tuyos. A mi **Anita**, porque lo primero que has hecho siempre al llegar a la Facultad ha sido venir a verme para ver cómo estaba. Aunque no lo creas, he aprendido mucho de ti dentro del animalario. Gracias por tenderme siempre tu mano cuando me has visto desbordada (y sin verme así) y por verme capaz de conseguir todo lo que me proponga. Tenemos que celebrar con una buena ración de chocolate con churros.

A todos los alumnos de Grado y Máster que han ido pasando por el laboratorio, especialmente **Mateo, Arley** y **Eva Bonilla**, por vuestra implicación como un miembro más del equipo en mis experimentos y por vuestras ganas de aprender siempre. Os habéis convertido también en compañeros y habéis sido un apoyo muy importante. También a todos los profesores y profesoras con los que he compartido horas de docencia durante varios cursos académicos, ha sido una experiencia enriquecedora y preciosa. Gracias a todos vosotros descubrí que también me gusta enseñar.

A mis psicofarmacólogos, **Jenni, Miguel, Carlos, Pedro**, porque con vosotros comenzó toda esta aventura.

*'I would like to thank everyone I met at the University of Oxford.* ***Dr. Daniel Anthony****, for welcoming me and letting me be part of your team. For promoting group plans and for those cheese and wine every week. I loved them!;* ***Dr. Daniel RS****, thank you for your teachings during this time. I admire your ability to reason and find solutions to problems. To my colleagues,* ***Tien****,* ***Izzy, Victor, Jacob*** *and* ***Listie****. I especially want to thank* ***Ross*** *and* ***Pippa*** *for all your support. I will never forget our Back Street Boys style "I want it that way" moment while doing PCRs, hahaha. And to* ***María****, who has been an absolute dream to meet and to work with. With you I simply can't say enough. I hope to see you all again one day, in Oxford or somewhere else!".*

Gracias también a mi estancia predoctoral en Oxford se formó la **banda de *New Marston***. No sólo habéis sido compañeros de piso, sino familia. Gracias a vosotros esta experiencia fue un sueño y tengo mi corazón cargado de recuerdos increíbles.

A mis amigos, que son como mi familia. **Helen**, **Ángela**, **Alejandro**, y ahora **Aya**, porque habéis vivido la Tesis conmigo de la mano. **Ana**, **Bárbara**, **Blanca**, **Ale**, **Marina**, **Pablo**, **Diego**, **Moha**, que sin tener muy claro qué hacía con mis animales siempre habéis estado para escucharme y apoyarme.

Gracias a toda mi familia. A mi **hermana**, por tu apoyo incondicional, tus ánimos, por tranquilizarme cuando la preocupación me desborda y por hacerme madrina de mi sobrino precioso **Gonzalo**. A mis cuñados, **Joni**, **Pablo** y **Álvaro**, y mis suegros **Blas** y **Belén**, por valorarme y estar para mí cuando lo necesito. A mi **tía Vicky**, porque eres mi tía y madrina favorita. A mi **madre**, por intentar siempre entender en qué consiste mi trabajo y creerme capaz de todo. A mi **padre**, porque, aunque no me puedas ver hoy finalizar esta etapa, estoy segura de que estás muy orgulloso de mi en el cielo. Tu siempre fuiste mi fan *number* 1.

A mi marido y compañero de vida, **Blas**. Tú, más que nadie, sabes cómo ha sido este camino. Sin ti sencillamente no habría sido posible. Gracias por creer siempre en mí, incluso cuando ni yo misma lo hago, y por apoyarme siempre en mis decisiones. Y a mi hija, **Vera**. Por ponerle el broche de oro a esta etapa.

Por último, gracias a **mí**. Por mi esfuerzo, mi perseverancia, mis ganas de aprender y mi resiliencia en estos años.

# FUENTES DE FINANCIACIÓN

Esta tesis doctoral se ha podido realizar gracias a la concesión de un contrato predoctoral para la formación de doctores 2019 (FPI2019) a su autora, procedente del Ministerio de Ciencia, Innovación y Universidades del Gobierno de España (PRE2019-087771) y se ha englobado dentro de dos proyectos de investigación, detallados a continuación:

1. **Title:** Signaling of bacterial products and neuroimmune alterations in alcohol abuse-induced neuropsychological impairments
   **Ref:** RTI2018-099535-B-100**.** Ministerio de Ciencia, Innovación y Universidades-Retos2018
   **Period of time**: 01/01/2019 - 31/12/2021
   **Principal Investigator:** Laura Orío.

2. **Title:** Peripheral and brain detoxification systems of bacterial toxins in alcohol use disorder and schizophrenia: the role of lipoproteins
   **Ref:** PID2021-127256OB-I00**.** Ministerio de Ciencia, Innovación y Universidades (PGC2021)
   **Period of time:** 01/09/22 – 31/08/26
   **Principal Investigator:** Laura Orío Ortiz

# INDEX

# 1. ABBREVIATIONS

**4-HNE:** 4-hydroxynonenal

**ABD:** alcohol binge drinking

**AD:** Alzheimer's Disease

**ADH:** alcohol dehydrogenase

**ALDH:** aldehyde dehydrogenase

**ANS:** autonomic nervous system

**ApoAI:** apolipoprotein AI

**ApoB:** apolipoprotein B

**ApoE:** apolipoprotein E

**ApoER2:** apolipoprotein E receptor 2

**Apos:** apolipoproteins

**AUD:** alcohol use disorder

**BBB:** blood brain barrier

**BELs:** blood ethanol levels

**BD:** binge drinking

**CD14:** cluster of differentiation 14

**CNS:** central nervous system

**COX-2:** cyclooxygenase 2

**CSF:** cerebrospinal fluid

**CXCL1:** C-X-C motif chemokine ligand 1

**DAMPs:** cell damage-associated molecular patterns

**EPM:** elevated plus maze

**FC**: frontal cortex

**GAPDH:** glyceraldehyde 3-phosphate dehydrogenase

**HDL:** high density lipoprotein

**HMGB1:** high mobility group box 1

**HPA:** hypothalamic-pituitary-adrenal axis

**IAC:** intensive alcohol consumption

**ICAM-1:** intercellular adhesion molecule 1

**IDL:** Intermediate-Density Lipoproteins

**i.g.:** intragastrically

**i.p.:** intraperitoneally

**IL-1β:** interleukin 1 beta

**IL-6:** interleukin 6

**IκB:** inhibitor of kappa B

**IκK:** inhibitor of κB kinase

**KO:** knock-out

**LBP:** lipopolysaccharide binding protein

**LDL:** low density lipoprotein

**LDLr:** low density lipoprotein receptor

**MAMPs:** microbe-associated molecules

**MDA:** malondialdehyde

**MWM:** morris water maze

**MyD88:** myeloid differentiation factor 88

**NOR:** novel object recognition

**OFT:** open-field test

**PAMPs:** pathogen-associated molecular patterns

**PBMCs:** peripheral blood mononuclear cells

**PFC:** prefrontal cortex

**PLTP:** phospholipid transfer protein

**PPRs:** Pattern Recognition Receptors

**PGE2:** prostaglandin E2

**SCFAs:** short-chain fatty acids

**SD:** standard drink

**SR-B1:** Scavenger receptor class B type 1

**TEDCA:** Test of Detection of Cognitive Impairment in Alcoholism

**TJs:** tight junctions

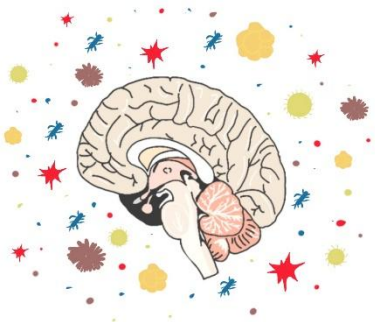
**TLR4:** toll-like receptor 4

**TNF-α:** tumour necrosis factor alpha

**VCAM-1:** vascular cell adhesion molecule 1

**ZO:** zonula occludens

# 2. RESUMEN Y ABSTRACT



## RESUMEN

**Título:** Papel de las apolipoproteínas y los componentes bacterianos en las alteraciones neuroconductuales inducidas por el consumo intensivo de alcohol.

**Introducción:** El consumo de alcohol en atracón (CAA) es un patrón de consumo intensivo de alcohol (CIA) cada vez más popular entre jóvenes y adultos que consiste en la ingesta de grandes cantidades de alcohol (5 unidades de bebida estándar en hombres y 4 en mujeres) en un período corto de tiempo (2 horas). Este patrón de consumo ha demostrado tener consecuencias muy relevantes en nuestro organismo, afectando al eje microbiota-intestino-hígado-cerebro y alterando la conducta durante la abstinencia. Además, el CAA predispone hacia el desarrollo de un Trastorno por Consumo de Alcohol.

Estudios previos han demostrado que el CIA altera la composición de la microbiota intestinal, produciendo disbiosis, y altera el entorno de estas bacterias al producir inflamación local en el intestino. Además, el CIA altera la estructura y función de la barrera intestinal, favoreciendo la translocación de bacterias *Gram-* y de componentes altamente inflamatorios, como el lipopolisacárido (LPS), componente de la membrana de dichas bacterias, desde el intestino hacia otros órganos o a la circulación sistémica. Estos procesos activan directamente nuestro sistema inmune innato, liderado por los receptores *Toll-like* 4 (TLR4), en células linfocitarias y en el hígado, desencadenando la liberación de moléculas proinflamatorias hacia la circulación sistémica, como citoquinas o moléculas asociadas a daño y muerte celular, promoviendo una inflamación periférica. El CIA también disminuye la expresión de uniones estrechas entre las células endoteliales de la barrera hematoencefálica (BHE), que protege a nuestro cerebro y lo aísla de componentes tóxicos periféricos, haciéndola más permeable al paso de sustancias proinflamatorias en estructuras como la corteza prefrontal (CPF). Además de esta activación del eje-microbiota-intestino-hígado-cerebro, el alcohol, por su naturaleza lipofílica, accede al cerebro y activa TLR4 y la cascada proinflamatoria en la CPF, con una fuerte activación glial y liberación de mediadores proinflamatorios. Esta neuroinflamación dependiente de la activación de TLR4 ha demostrado que está implicada en las alteraciones emocionales y cognitivas durante la abstinencia al alcohol. Tanto los mecanismos periféricos (activación del nervio vago por citoquinas inflamatorias, disrupción de barreras biológicas, etc.) como los centrales (acción directa del alcohol en el cerebro) contribuyen al desarrollo de esta neuroinflamación y alteraciones conductuales tras el CIA. Se desconoce si la disfunción de la BHE inducida por el CIA podría permitir el paso de componentes bacterianos a ciertas estructuras cerebrales, amplificando la neuroinflamación. La mayoría de los estudios científicos hasta la fecha han sido realizados en roedores machos, por lo que es necesaria una investigación integral teniendo en cuenta la perspectiva de sexo.

Las apolipoproteínas (Apos) han emergido en los últimos años por su posible implicación en procesos inflamatorios y cognitivos, y se han relacionado con alteraciones neuropsiquiátricas, como la enfermedad de Alzhéimer. Las Apos son la parte proteica de las lipoproteínas, y algunas de ellas, como la ApoAI, ApoB o ApoE, se integran en diferentes proporciones en las lipoproteínas de baja (LDL) o alta (HDL) densidad. Las HDL y su principal Apo, la ApoAI, se unen al LPS para su aclaramiento en el hígado, ejerciendo un efecto potencialmente antiinflamatorio a nivel periférico. El LPS también puede unirse a otras Apos, dependiendo del estado inflamatorio del organismo. Nuevas evidencias parecen indicar que algunas Apos podrían también estar unidas al LPS formando agregados en estructuras cerebrales cercanas a los órganos de comunicación cerebro-periferia, como los órganos circunventriculares, en condiciones fisiológicas. Los efectos de estas Apos y su posible unión a LPS en el cerebro, así como su influencia en la neuroinflamación y las alteraciones emocionales/cognitivas en machos y hembras tras el CIA son desconocidos.

**Objetivos:** Caracterizar la presencia de componentes del LPS, como el Lípido A y el Core, en su forma libre o unido a Apos específicas, en la CPF y el cerebelo de animales macho y hembra expuestos a CIA y comprender su relación con la neuroinflamación y las alteraciones neuroconductuales durante la abstinencia temprana, con una perspectiva de sexo. **Hipotetizamos** que el LPS podría estar unido a Apos en estructuras cerebrales donde existe disrupción de la BHE, contribuyendo a la neuroinflamación y alteraciones conductuales tras el CIA, y con diferencias por sexo, o bien ser un mecanismo antiinflamatorio de respuesta.

**Métodos:** se empleó un modelo animal de CAA con ratas Wistar machos y hembras, en el que se modelizó el CIA mediante administraciones intragástricas repetidas de alcohol, durante 4 días consecutivos, y se realizaron pruebas de Western blot, co-inmunoprecipitación, ensayos de inmunoadsorción ligado a enzima (ELISA) y PCR cuantitativa en tiempo real (qPCR) para determinar componentes inmunes, proinflamatorios, expresión de Apos y/o agregados con LPS. En los estudios conductuales se administró un pretratamiento con HDL (i.p., 20 mg/Kg) o el péptido mimético 4F (i.p., 5 mg/Kg), antes de cada dosis de alcohol (en un protocolo de 4 días no consecutivos) como estrategia para potenciar la acción biológica de la ApoAI. Se aplicaron pruebas comportamentales de ansiedad (EPM/OFT), depresión (Porsolt), anhedonia (SPT) y memoria (MWM/NOR).

**Resultados**: Los animales machos tratados con el protocolo de CIA, pero no las hembras, mostraron niveles de alcohol en sangre tipo *binge* (>0.08 g/dL) a las 3h tras el último atracón de alcohol. Las hembras no mostraron niveles de atracón en esa ventana temporal, pero mostraron

un aumento en plasma de los niveles de corticosterona tras CIA, en relación a sus controles, que no se observó en los machos. Se observaron diferencias en la expresión de las enzimas hepáticas involucradas en el metabolismo del alcohol: las hembras control mostraron más expresión de ALDH de forma basal, comparadas con machos control y una disminución de esta enzima tras el CIA. Las hembras tratadas con alcohol mostraron mayores niveles plasmáticos de ApoAI que sus controles y también se observaron diferencias sexuales en los niveles fisiológicos de LDL y ApoB en plasma, que fueron mayores en los controles macho que en las hembras control. Tanto machos como hembras tratadas con alcohol mostraron aumentos en los niveles plasmáticos de LBP, indicativo de inflamación periférica. A nivel cerebral, no se encontraron diferencias en la expresión de Lípido A y/o Core o de Apos (ApoAI, B o E) libres en la CPF o cerebelo tras el CIA tanto en machos como en hembras. Sin embargo, el Lípido A formó agregados con ApoAI en hembras y con ApoB en machos en la CPF tras el CIA, pero no en el cerebelo. No se encontraron agregados cerebrales con la ApoE, pero el CIA aumentó los niveles de ApoER2 en el hemisferio cerebelar y de LDLr en la CPF sólo en los machos. Además, en la CPF, se observó una sobreexpresión de TLR4 sólo en los machos tratados con alcohol, efecto que no fue observado en hembras tras el CIA. A nivel conductual, las hembras tratadas con alcohol mostraron un comportamiento desinhibido en el EPM, contrario a lo observado en estudios previos en machos. Sin embargo, tuvieron un comportamiento ansioso en el OFT, así como de tipo depresivo y anhedónico. A nivel cognitivo, el CIA promovió en las hembras un leve déficit en la memoria de reconocimiento a largo plazo, pero no en el aprendizaje o la memoria espacial, durante la abstinencia temprana. El pretratamiento con HDL antes de cada *binge* en hembras no mejoró las alteraciones inducidas por el alcohol, incluso produjo deficiencias en el aprendizaje en MWM, agravó la memoria de reconocimiento, el estado ansioso y la anhedonia. Sorprendentemente, agravó la memoria espacial en hembras control. Además, promovió un estado inflamatorio en plasma y en la CPF, independientemente del efecto del alcohol. De forma similar, el pretratamiento con el péptido 4F, mimético de ApoAI, antes de cada *binge* produjo efectos significativos perjudiciales en el aprendizaje en MWM, además de agravar la memoria espacial en hembras control.

**Conclusión:** determinadas Apos parecen estar agregadas a componentes específicos del LPS, como el Lípido A, en la CPF, pero no en el cerebelo, tras un CIA y de una manera dependiente de sexo. Específicamente, el lípido A se ha encontrado unido con ApoAI en hembras y con ApoB en machos, sugiriendo una posible diferencia sexual en la formación de estos agregados. Las hembras CIA tienen niveles plasmáticos más elevados de ApoAI y no muestran elevaciones de TLR4 en la CPF, a diferencia de los machos tratados con alcohol, que muestran una regulación de TLR4 en esta estructura, lo que podría sugerir que el agregado con ApoAI es un mecanismo de detoxificación de LPS en el cerebro. Sin embargo, la potenciación de la ApoAI

a través de un pretratamiento inespecífico con HDL parece inducir fenómenos de inflamación en la CPF tanto en hembras CIA como control, que se corresponden con un peor desempeño en tareas conductuales en ambos grupos experimentales. De forma similar, el pretratamiento más específico con el péptido 4F no mejoró las alteraciones conductuales tras el CIA en hembras e incluso empeoró el aprendizaje en ambos grupos experimentales y la memoria espacial en controles, lo que sugiere que la ApoAI podría ser un mecanismo de trasporte de componentes bacterianos al cerebro en hembras. Este estudio señala importantes diferencias sexuales en la relación entre diversas Apos y la neuroinflamación inducida por el CIA, que podrían estar relacionadas con las alteraciones conductuales durante la abstinencia.

## ABSTRACT

**Title:** Role of apolipoproteins and bacterial components in neurobehavioral alterations induced by intensive alcohol consumption.

**Introduction:** Alcohol binge drinking (ABD) is an increasingly popular pattern of intensive alcohol consumption (IAC) among adolescents and adults, consisting in the consumption of large amounts of alcohol (5 standard drinking units for men and 4 for women) in a short period of time (2 hours). This drinking pattern has been shown to have very relevant consequences for our organism, affecting the microbiota-gut-liver-brain axis and altering behaviour during abstinence. In addition, ABD predisposes to the development of alcohol use disorder.

Previous studies have shown that ABD alters the composition of the gut microbiota, leading to dysbiosis, and alters the environment of these bacteria by producing local inflammation in the gut. In addition, ABD alters the structure and function of the intestinal barrier, favouring the translocation of *Gram-* bacteria and highly inflammatory components, such as lipopolysaccharide (LPS), a component of the membrane of these bacteria, from the gut to other organs or into the systemic circulation. These processes directly activate our innate immune system, led by Toll-like receptors 4 (TLR4), in lymphocyte cells and in the liver, triggering the release of pro-inflammatory molecules into the systemic circulation, such as cytokines or molecules associated with cell damage and death, promoting peripheral inflammation. IAC also decreases the expression of tight junctions between endothelial cells of the blood-brain barrier (BBB), which protects our brain and insulates it from peripheral toxic components, making it more permeable to the passage of pro-inflammatory substances in structures such as the prefrontal cortex (PFC). In addition to this activation of the microbiota-gut-liver-brain axis, alcohol, due to its lipophilic nature, access to the brain and activates TLR4 and the pro-inflammatory cascade in the PFC, with strong glial activation and release of pro-inflammatory mediators. This TLR4 activation-dependent neuroinflammation has been shown to be involved in emotional and cognitive disturbances during alcohol abstinence. Both peripheral (activation of the vagus nerve by inflammatory cytokines, disruption of biological barriers, etc.) and central (direct action of alcohol on the brain) mechanisms contribute to the development of this neuroinflammation and behavioural alterations after ABD. It is not known whether alcohol-induced BBB dysfunction could allow the passage of bacterial components into certain brain structures, promoting neuroinflammation. Most studies to date have been conducted in male rodents, so there is a need for a comprehensive sex-specific study.

Apolipoproteins (Apos) have emerged in recent years due to their possible involvement in inflammatory and cognitive processes, and have been linked to neuropsychiatric disorders, such as Alzheimer's disease. Apos are the protein part of lipoproteins, and some of them, such as ApoAI, ApoB or ApoE, are integrated in different proportions in low-density lipoproteins (LDL) or high-density lipoproteins (HDL). HDL and its main Apo, ApoAI, bind to LPS for clearance in the liver, exerting a potentially anti-inflammatory effect at the peripheral level. LPS may also bind to other Apos, depending on the inflammatory state of the body. New evidence suggests that some Apos may also be bound to LPS forming aggregates in brain structures close to the brain-peripheral communicating organs, such as the circumventricular organs, under physiological conditions. The effects of these Apos and their possible binding to LPS in the brain, as well as their influence on neuroinflammation and emotional/cognitive alterations in males and females after IAC are unknown.

**Objectives:** To characterise the presence of LPS components, such as Lipid A and Core, in its free form or bound to specific Apos, in the PFC and cerebellum of male and female animals exposed to IAC and to understand their relationship with neuroinflammation and neurobehavioural alterations during early abstinence, with a sex perspective. We **hypothesised** that LPS may be bound to Apos in brain structures where BBB disruption is present, contributing to neuroinflammation and behavioural changes after IAC with sex differences, or may be an anti-inflammatory response mechanism.

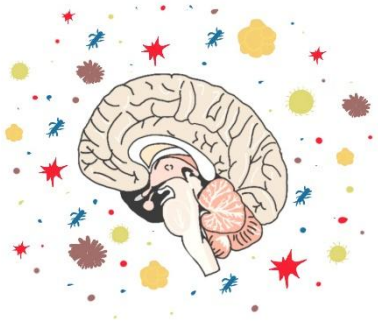
**Methods:** An animal model of ABD was used in male and female Wistar rats, in which IAC was modelled by repeated intragastric administration of alcohol for 4 consecutive days, and Western blotting, co-immunoprecipitation, enzyme-linked immunosorbent assay (ELISA) kits and real-time quantitative PCR (qPCR) were performed to determine immune components, pro-inflammatory components, Apos expression and/or LPS aggregates. In the behavioural studies, pre-treatment with HDL (i.p., 20 mg/kg) or the peptide mimetic 4F (i.p., 5 mg/kg) was administered prior to each alcohol administration (in a 4-day non-consecutive protocol) as a strategy to enhance the biological effect of ApoAI. Behavioural tests of anxiety (EPM/OFT), depression (Porsolt), anhedonia (SPT) and memory (MWM/NOR) were used.

**Results:** ethanol-treated males, but not females, showed binge drinking blood alcohol levels (>0.08 g/dL) at 3h after the last alcohol binge. Females did not show binge levels at the time of blood extraction but showed an increase in plasma corticosterone levels after CIA, in comparison to their controls, which was not observed in males. Differences in the expression of liver enzymes involved in alcohol metabolism were observed: control females showed higher expression of ALDH at baseline compared to control males and a decrease in this enzyme after

IAC. Ethanol-treated females showed higher plasma ApoAI levels than their controls and basal sexual dimorphisms were also observed in plasma LDL and ApoB levels, which were higher in male controls than in female controls. Both ethanol-treated males and females showed increases in plasma LBP levels, indicative of peripheral inflammation. In the brain, no differences were found in the expression of free Lipid A and/or Core or Apos (ApoAI, B or E) in the PFC or cerebellum after IAC in both males and females. However, Lipid A formed aggregates with ApoAI in females and with ApoB in males in the PFC after IAC, but not in the cerebellum. No brain aggregates were found with ApoE, but IAC increased ApoER2 levels in the cerebellar hemisphere and LDLr in the PFC only in males. Additionally, TLR4 overexpression was observed only in ethanol-treated males in the PFC, an effect that was not observed in females after IAC. At the behavioural level, ethanol-treated females revealed disinhibited-like behaviour in the EPM, contrary to what was observed in previous studies in males. However, they showed anxiety in the OFT, as well as depressive-like behaviour and anhedonia after IAC. At the cognitive level, IAC promoted a slight deficit in long-term recognition memory in these females, but not in learning or spatial memory, during early abstinence. Pre-treatment with HDL before each binge in females did not ameliorate alcohol-induced impairments, even produced learning deficits in MWM, aggravated recognition memory, anxiety-like behaviour and anhedonia. Surprisingly, it aggravated spatial memory in control females. In addition, it promoted an inflammatory state in plasma and PFC, disregarding of alcohol effect. Similarly, pre-treatment with the ApoAI mimetic peptide 4F before each binge produced significant detrimental effects on learning in MWM, as well as aggravating spatial memory in control females.

**Conclusions:** certain Apos appear to be aggregated to specific LPS components, such as Lipid A, in the PFC, but not in the cerebellum, after an IAC and in a sex-dependent manner. Notably, Lipid A has been found bound to ApoAI in females and to ApoB in males, suggesting a possible sex difference in the formation of these aggregates. Ethanol-treated females have higher plasma levels of ApoAI and do not show elevations of TLR4 in the PFC, unlike ethanol-treated males, who show up-regulation of TLR4 in this structure, which may suggest that ApoAI aggregation is a mechanism of LPS detoxification in the brain. However, ApoAI potentiation through non-specific pre-treatment with HDL seems to induce inflammatory effects in the PFC in both ethanol and control females, corresponding to worse performance in behavioural tasks in both experimental groups. Similarly, more specific pre-treatment with 4F peptide did not improve behavioural alterations after IAC in females and even worsened learning in both experimental groups and spatial memory in controls, suggesting that ApoAI could be a mechanism of transport of bacterial components to the brain in females. This study points to important sex differences in the relationship between various Apos and alcohol-induced neuroinflammation, which could be related to behavioural alterations during abstinence.

# 3. INTRODUCTION



## 3.1 Alcohol: drug of abuse

Alcohol is one of the most widely consumed substances in contemporary society and its consumption is widely integrated in our society. Alcohol is a psychoactive substance with the capacity to induce intoxication and dependence and its consumption. Even at low levels, it is associated with an inherent health risk, but the majority alcohol-related harms such as injuries, alcohol use disorders (AUD), liver disease, cancer and/or cardiovascular diseases are attributable to heavy episodic or continuous alcohol consumption. However, it is also one of the most preventable causes of disease and mortality. According to the World Health Organization (WHO), the global average consumption per person in 2019 was 8.2 L/year per capita in case of men and 2.2 L/year in women. In that year, alcohol was identified as the cause of almost 2.6 million of deaths in the world. The adverse effects of alcohol consumption are disproportionately concentrated among younger individuals, with the highest percentage (13%) of deaths attributable to alcohol consumption in 2019 occurring among those aged 20-39. The data on global alcohol consumption indicate that approximately 400 million individuals aged 15 and above are affected by an Alcohol Use Disorder (AUD), with an estimated 209 million individuals exhibiting symptoms of alcohol dependence (World Health Organization, 2024). In Spain, alcohol is the psychoactive substance with the highest prevalence of use (92.9%) in 2024, with an average age of consumption starting at 16 for males and 17 for females (EDADES, 2024).

### 3.1.1 Binge drinking

Alcohol binge drinking (ABD) is one of the most widespread patterns of alcohol consumption worldwide among adolescent people and is becoming increasingly prevalent among older adults. According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA) in the United States, it consists of an intensive alcohol consumption (IAC) that brings blood ethanol concentrations (BELs) to $\geq$ 0.08 g/dL in a short period of time. It means 5 or more alcoholic beverages or standard drink (SD) for males and 4 or more in females in approximately two hours (Jones et al., 2018; Kuntsche et al., 2017; Maurage et al., 2020). The recurrence of such episodes of excessive alcohol consumption results in an alternation between periods of intense alcohol intoxication and periods of abstinence. There are slight variations in both the number of drinks consumed and the time frame within which they are consumed between countries. The SD for each type of alcoholic beverage is calculated by considering the grams of alcohol in each beverage. In Spain, the SD is defined as 10 grams of alcohol. This is approximately equivalent to the typical volume of a 100 mL glass of wine with 13% alcohol by volume, a 300 mL glass of beer with 4% alcohol by volume, or 30 mL of liquor with 40% alcohol by volume (Spanish Ministry of Health, 2021).

The prevalence of binge drinking is higher in Nordic and Baltic countries due to the prevalence of an episodic but intense drinking culture and how it is integrated into everyday life, in comparison to southern Europe, where consumption is more moderate and associated with meals, thus reducing alcohol-related risks (Helasoja et al., 2007). However, ABD has gained considerable popularity in recent decades in Spain, particularly among the younger generation. The beverage was promoted for consumption in large quantities at festivals, diverging from the more leisurely and gastronomic consumption patterns that were traditionally observed in our culture (Llamosas-Falcón et al., 2022).

While the total quantity of alcohol consumed may be comparable to other patterns, the associated health risks and social consequences are significantly elevated due to the temporal concentration of consumption. Moreover, there are no pauses between drinks, nor is there any accompaniment of food. Intake is rapid and uncontrolled, and the group environment serves to encourage this behaviour (NIAAA).

ABD consumption produces acute and chronic effects. Alcohol poisoning, blackouts or violence are short-term effects. However, ABD can also cause long-term health effects, including alterations in the structure and/or function of the central nervous system (CNS), including loss of white matter and behavioural alterations such as cognitive impairment, memory loss, learning problems or even the death. Anyone can be susceptible to its deleterious effects, especially adolescents, due to their immature prefrontal cortex (PFC), which is responsible for executive functions and impulse control (F. T. Crews et al., 2016; F. T. Crews & Vetreno, 2014; De La Monte & Kril, 2014; Fan et al., 2023). Many of these changes during adolescence may remain into adulthood in rat models (Vetreno & Crews, 2012) and repeated consumption of alcoholic beverages may lead to the development of AUDs.

All these effects of IAC can affect organs such as the brain, intestine and liver, key components in the proper functioning of the gut-liver-brain axis, as it is explained in the following sections.

## 3.2 Microbiota-gut-liver-brain axis: effects of binge drinking

The complex communication between the gut microbiota, the liver and the brain, commonly referred to as the "microbiota-gut-liver-brain axis", is of vital importance in maintaining overall physiological homeostasis. This axis has shown to have significant implications in neuropsychiatric pathologies, as alterations in the composition of the gut

microbiota (dysbiosis) and increased gut permeability can trigger systemic and neuroinflammatory responses, contributing to the development of psychiatric disorders such as anxiety, depression and schizophrenia (Cryan & Dinan, 2012). Furthermore, in the context of alcohol addiction, chronic alcohol consumption has been demonstrated to alter the microbiota, increase intestinal permeability (termed 'leaky gut') and promote increased liver and systemic inflammation. This, in turn, affects the dopaminergic and reward pathways in the brain, thereby perpetuating alcohol dependence and exacerbating associated psychiatric disorders (Leclercq et al., 2014; Stärkel et al., 2018). In recent years, it has also been shown that IAC disrupt the homeostasis of this axis, promoting gut dysbiosis, altering the integrity of biological barriers and causing neuroinflammation, with cognitive and/or emotional alterations (Antón et al., 2017, 2018). The effects of ABD are explained in the following sections.

### 3.2.1 Intestinal microbiota: dysbiosis

The microbiota is the set of microorganisms that live in symbiosis with the human body. The presence of these microorganisms is of vital importance as they enable the body to maintain a healthy equilibrium between health and disease. In particular, the human gastrointestinal tract contains a vast number of bacteria, with an estimated $10^{12}$ bacteria per gram and over 500 different species, mostly bacteria but also viruses, fungi, protozoa and other microorganisms (Cryan et al., 2019; Vassallo et al., 2015).

The interaction between the gut microbiota and the host is mutually beneficial. While the precise roles of the microbiota remain largely unknown, several functions have been identified. The host provides a living environment and nutrients for the microbiota, while the bacteria contribute by defending against pathogens, taking part in digestion processes, supporting metabolic processes, supplying essential nutrients like vitamins, and enhancing immune system function (Rea et al., 2016; Stärkel et al., 2018; Vassallo et al., 2015).

The microbiota-gut-liver-brain axis represents a network of connections between tissues and organs, including the brain, glands, gut, immune cells, liver and gastrointestinal microbiota. These components participate in a complex, multidirectional communication to maintain homeostasis and are controlled by the autonomic nervous system (ANS) via the vagus nerve. The vagus nerve facilitates the transmission of information from the gut microbiota to the CNS, thereby eliciting a physiological response. The neuroendocrine system, including the hypothalamic-pituitary-adrenal axis (HPA), plays also a role in gut-brain communication. This system is crucial for the body's ability to adapt to stress, regulate behaviour and maintain mood and regulates cortisol secretion. Cortisol can affect immune cells (including cytokine secretion)

both locally in the gut and systemically (Cryan & Dinan, 2012; Dalton et al., 2019; Grenham et al., 2011; Zheng et al., 2023).

The microbiota may be also a source of pro-inflammatory molecules and can modulate local immune responses at the epithelial level. The immune system is responsible for regulating the physiological process of inflammation, which is the body's natural defence mechanism against pathogens and damage. One of the biological alterations associated with the inflammatory process is the release of cytokines, which are proteins that modulate the inflammatory response. Interleukin (IL)-1β, IL-6 and tumour necrosis factor α (TNF-α) are amongst the most widely investigated proinflammatory cytokines. In addition, microbiota can produce and release neurotransmitters, such as serotonin or dopamine, and short-chain fatty acids (SCFAs), such as butyric acid or lactic acid, derived from the fermentation of polysaccharides. These compounds are secreted into the gut lumen and transported across the epithelial barrier into the bloodstream, where they interact with the host immune system. They can also exert a localised effect on the enteric nervous system and the vagus nerve, which transmits signals directly to the brain (Getachew et al., 2024; Morais et al., 2021; Rea et al., 2016).

Disruptions in the gut microbiota-brain axis have been observed to trigger a wide range of physiological and behavioural changes, including activation of the HPA axis and the secretion of glucocorticoids, as well as alterations in neurotransmitter system activity and immune function (Cryan et al., 2019; Cryan & Dinan, 2012; Rea et al., 2016; Rodríguez-González et al., 2021). Alterations in this axis have also been closely related with ABD (Antón et al., 2018; Rodriguez-Gonzalez & Orio, 2020; Stärkel et al., 2018) and AUD (Gorky & Schwaber, 2016).

It has been demonstrated that ABD can influence the composition of the gut microbiota (Rodríguez-González et al., 2021). This phenomenon alters the equilibrium between beneficial and detrimental bacteria, which is a logical consequence given that alcohol is also a foodstuff, ingested and subsequently passing through the gastrointestinal tract. This results in a less healthy microbiota profile (dysbiosis) and affects the surrounding environment of the bacteria. Intestinal bacteria can alter their behaviour in response to the surrounding environment, including local intestinal inflammation.

The impact of alcohol consumption on the gut-brain axis is associated with the impairment of the intestinal barrier integrity and the pro-inflammatory effects of alcohol on the intestinal barrier itself, as it is explained in the next section. In addition, several studies have reported that different patterns of alcohol consumption have the capacity to induce gut dysbiosis by altering the composition and/or activity of the microbiota:  moderate ethanol consumption in

both humans (Kosnicki et al., 2019) and animals (Segovia-Rodríguez et al., 2022); AUD in humans (Leclercq et al., 2014; Litwinowicz et al., 2020); ABD in laboratory animals (E. Mutlu et al., 2009; Rodríguez-González et al., 2021; Vetreno et al., 2021). The effects of alcohol on gut microbiota can vary depending on the species studied (humans, mice, rats) and the specific alcohol protocol employed. However, alcohol generally appears to increase bacteria from the phyla Proteobacteria, Firmicutes, and/or Actinobacteria (E. A. Mutlu et al., 2012; Vetreno et al., 2021), while reducing those from the phylum Bacteroidetes (E. A. Mutlu et al., 2012; Phillip A Engen et al., 2015). Other studies have also reported a decrease in Firmicutes (Kosnicki et al., 2019; Rodríguez-González et al., 2021).

These changes may significantly influence immune system activation, particularly because Proteobacteria, as *Gram*-negative bacteria, are potent activators of Toll-like receptor 4 (TLR4). These receptors are in the periphery and the brain and are a key component of the innate immune system that triggers inflammatory responses and activates the adaptive response to pathogens.

#### 3.2.1.1 Bacterial Lipopolysaccharide (LPS)

Alcohol-induced gut dysbiosis, combined with an increase of bacteria from the phyla Proteobacteria (see previous section 3.2.1) and a compromised intestinal barrier, facilitates the translocation of bacterial components, such as lipopolysaccharide (LPS), into the systemic circulation. This molecule is of special relevance to the context of this Doctoral Thesis.

LPS is a large component of the outer membrane of *Gram*-negative bacteria (Erridge et al., 2002). It is an amphipathic molecule composed of three different parts attached to each other (Figure 1): Lipid A, a glycolipid domain that determines toxicity and inflammation mainly by activating TLR4; the Core, a short chain of sugar residues; and the O-antigen, a highly variable polysaccharide moiety. Thus, not all endotoxins are the same. The toxicity of LPS varies based on the composition of Lipid A, which is influenced by the bacterial species, strain, and environmental conditions, and the O-antigen, which determines the antigenicity of endotoxin (Brown, 2019; Kumar et al., 2024; Lorenzo et al., 2019). For example, the Lipid A moiety from Enterobacteriaceae, such as *E. coli*, promotes a strong pro-inflammatory signal due to it has 6 fatty acyl chains, activating TLR4 and leading to a high expression of cytokines such as IL-1β (Netea et al., 2002). However, Lipid A structures synthesized by other families of bacteria with less fatty acyl chains, such as the cyanobacteria *Rhodobacter sphaeroides*, do not induce cytokine expression in humans and rodents since their binding to TLR4 is considered weak, functioning as an antagonist of this receptor (Anwar et al., 2015). Furthermore, some studies have shown that

prolonged exposure to LPS could induce a state of tolerance that reprograms the inflammatory response, resulting in a reduction in the production of inflammatory cytokines. This phenomenon is known as endotoxin tolerance (Seeley & Ghosh, 2017).

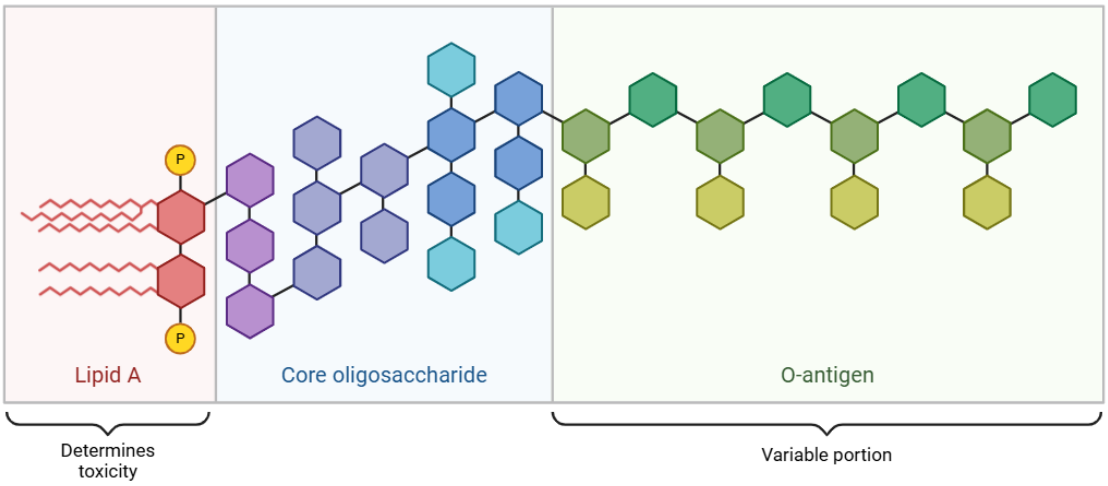



**Figure 1. General structure of LPS**. It is part of the wall of *Gram*- bacteria and consists of a glycolipid part (Lipid A), a short chain of sugar residues (Core) and a variable polysaccharide moiety (O-antigen) *(Modified from BioRender).*

*Gram*-negative bacteria are prevalent in the mammalian gut, particularly in the lower intestine, but they are also found in saliva, dental plaque, skin, respiratory tract and urinary tract (Sender et al., 2016). Under physiological conditions, low circulatory levels of LPS have been detected in the plasma of animals (Martín-Hernández et al., 2016; Topchiy et al., 2016) and humans (Brown, 2019), suggesting that minimal quantities of LPS constantly pass through the intestinal epithelial barrier. LPS could permeate through the healthy GI barrier into the portal circulation to the liver, where it is neutralised. However, in the presence of a leaky gut, for example due to abusive alcohol consumption, the high levels of LPS are not efficiently cleared in the liver, resulting in elevated LPS levels in the circulation, producing an excessive cellular response that can induce damage to the host both in human (Orio et al., 2018) and animals (Antón et al., 2017, 2018). Thus, endotoxin-inactivating processes are of extreme importance (R. Rao, 2009; Szabo & Bala, 2010). LPS-induced cellular responses are the net result of the interaction of LPS with various plasma components, such as soluble cluster of differentiation 14 (CD14), LPS-binding protein (LBP), phospholipid transfer protein (PLTP) and membrane receptors, such as membrane-bound CD14 and TLR4 (Ciesielska et al., 2021). Balanced cellular responses are essential for the host defence against bacterial infections (Lorenzo et al., 2019). There are several routes of LPS detoxification in the circulation, but the most relevant is by its incorporation into lipoproteins (Phillips, 2013), as it is explained in the section 3.4.4.1.

### 3.2.2 Intestinal barrier and local inflammation

The intestine represents the primary point of interaction between the organism and the external environment. It is surrounded by the intestinal barrier, which is one of the most extensive in mammals, and its principal function is to act as a physical and biochemical barrier, facilitating the absorption of nutrients and preventing the infiltration of substances and pathogens from the lumen into the internal environment. The maintenance of a stable intestinal barrier is dependent on the gut microbiome, the host immune system and the intestinal epithelium (Di Vincenzo et al., 2024; Maldonado-Contreras & McCormick, 2011).

The integrity of the intestinal epithelium is of vital importance for maintaining the functionality of the intestinal barrier, where any molecule and/or nutrient can be absorbed through the epithelium by specific transport routes, such as endocytosis. The mucus layer represents the **initial line of defence** for the intestinal barrier, exerting a regulatory effect on autochthonous bacterial communities, preventing their excessive growth and expansion. The **second line of defence** is constituted by enterocytes, which are the principal epithelial cells that constitute the intestinal barrier. They are equipped with binding proteins, including tight junctions (TJs), which are formed by transmembrane proteins (occludin and claudin) and cytoplasmic proteins (zonula occludens (ZO)). These TJs play a pivotal role in maintaining the structural and functional integrity of the epithelial barrier, facilitating the attachment of adjacent epithelial cells and preventing bacterial epithelial invasion and translocation, as well as the stimulation of immune cells in the lamina propria. The **third line of defence** is the immune system. The enterocytes express innate immune receptors, mainly Toll-like receptor 4 (TLR4), and can release inflammatory cytokines and chemokines, which will be discussed in greater detail later on (Di Vincenzo et al., 2024; Maldonado-Contreras & McCormick, 2011; Stärkel et al., 2018; Turner, 2009).

The immune system can be further subdivided into innate immunity and acquired immunity. Both components of the immune system identify invading microorganisms as non-self, which initiates an immune response to eliminate them. Throughout this Thesis, we focus on the innate immune system, which is mediated by several receptors, including those belonging to the family of Pattern Recognition Receptors (PRRs), such as Toll-like receptor (TLR) (García Bueno et al., 2016; Takeda & Akira, 2005).

At least thirteen different types of TLRs have been described in the scientific literature. Notably, TLR1-9 have been identified as being conserved between humans and rodents (Shastri et al., 2013; Takeda & Akira, 2005) and some of them have been observed to be overexpressed

in the FC of individuals who have died from alcohol use disorder (AUD) as well as in the cortex of mice that have been chronically treated with alcohol (F. T. Crews et al., 2013). It is well-known that alcohol consumption induces peripheral and central inflammatory response, activating the innate immune system by the TLR4 receptor. These receptors can recognise a range of potentially harmful molecules, including alcohol (Alfonso-Loeches et al., 2010; Orio et al., 2019), cell damage-associated molecular patterns (DAMPs), which are endogenous substances released during tissue damage such as HMGB1, pathogen-associated molecular patterns (PAMPs), expressed by microbial invaders such as LPS, and microbe-associated molecules (MAMPs) (Caputi & Giron, 2018; R. Wu & Li, 2020).

In particular, TLR4 is widely expressed in the organism, not only in the periphery but also in the brain, mainly by microglia, astrocytes and neurons. TLR4 is relevant in this Doctoral Thesis because its main ligands are alcohol and LPS, as it has been explained before. Some LPS-binding proteins participate in LPS-TLR4 binding. Among those, LPS-binding protein (LBP) and CD14 are essential for LPS recognition by TLR4 and lymphocyte antigen 96 (MD-2) (C. C. Lee et al., 2012; Tsukamoto et al., 2010). The inflammatory cascade has been well-described by some authors (Antón et al., 2017; Orio et al., 2019; Toledo Nunes et al., 2019; Vetreno & Crews, 2012).

Briefly, when TLR4 is activated by alcohol and/or LPS, it activates its adaptor-dependent, which is myeloid differentiation factor 88 (MyD88). This initiates a sequence of processes that result in the phosphorylation of the inhibitor of *kappa* B (IκB) protein complex by the inhibitor of κB kinase (IκK), leading to degradation of IκB and inducing the translocation of nuclear factor *kappa* B (NF-κB) transcription factor to the nucleus. Once NF-κB is in the nucleus, transcription of some pro-inflammatory genes is induced, such as cytokines (TNF-α), interleukins (IL-1β and IL-6) or enzymes such as cyclooxygenase 2 (COX). This produces oxidative/nitrosative stress, increasing the synthesis of lipid peroxidation bioproducts, such as 4-hydroxynonenal (4-HNE) and/or malondialdehyde (MDA). They cause cell instability and cell death, particularly through the activation of caspases, and in response to damage tissue, HMGB1 can be released, activating once again TLR4 and amplifying the inflammatory response in a vicious cycle (See Figure 2).

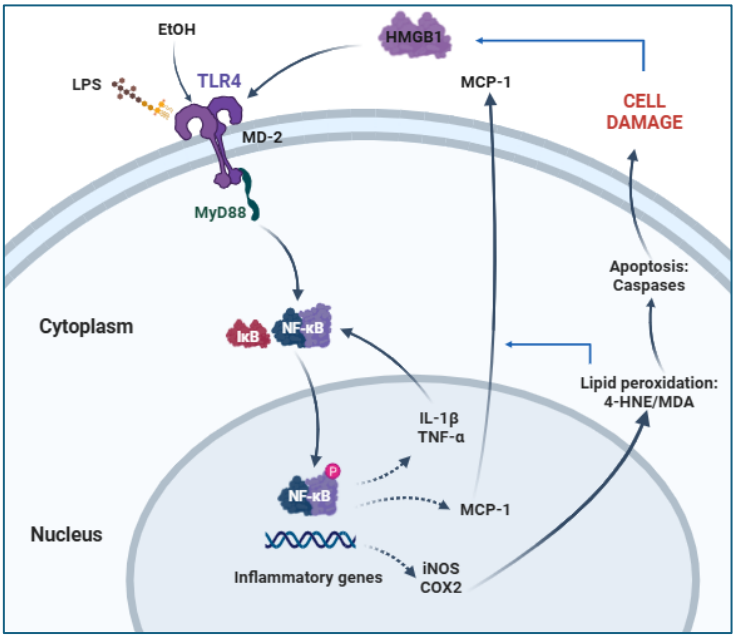



**Figure 2. Schematic of the Toll-like receptor 4 signalling pathway** *(adapted from Orio et al., 2019)*. EtOH, as well as LPS (PAMPs) or HMGB1 (DAMPs) are direct agonist of TLR4. *ETOH*=ethanol, *LPS*=lipopolysaccharide; *MD-2*=lymphocyte antigen 96; MyD88=myeloid differentiation primary response 88; *NF-κB*=nuclear factor *kappa* B; *NF-κB-P*=phosphorylated nuclear factor *kappa* B; IκB=inhibitor of κB; *IL-1β*= interleukin 1 beta; *TNF-α*=tumour necrosis factor alpha; *MCP-1*=monocyte chemoattractant protein-1; *iNOS*=inducible NO synthase; *COX2*=cyclooxygenase 2; *4-HNE*=4-hydroxynonenal; *MDA*= malondialdehyde; *HMGB1*=high mobility group box 1 protein.

The permeability and thus the function of this intestinal barrier can be compromised in certain physiological conditions, including in response to stress, infection or diet (Konturek, 2011; Rohr et al., 2020). In addition, recent studies in animals and patients have shown that different alcohol patterns of consumption can also disrupt the intestinal epithelial barrier (Bishehsari et al., 2017; Stärkel et al., 2018), resulting in increased gut permeability: in AUD in humans (Leclercq et al., 2012, 2014); chronic exposure in animals (Keshavarzian et al., 2009); chronic ABD (Fonseca-Pereira et al., 2024) and ABD in animals (Antón et al., 2018). Alcohol has been shown to alter the epithelial TJs integrity, decreasing the levels of mRNA and TJ-forming proteins, including occludin and claudin. This results in a dilation of the intercellular distance of the TJ. It is notable that research examining the impact of intensive alcohol consumption on intestinal permeability is limited. Thus, Antón et al studied the decrease in TJs by various techniques including electron microscopy, where dilatations in the intestinal barrier were observed (Figure 3). Moreover, they examined the potential impact of a reduction in TJs following ABD on intestinal barrier function. Thus, animals that underwent ABD showed bacterial growth beyond the barrier, in the mesenteric lymph nodes, which is the first route of entry, and spleen but not in

the liver and blood, indicating for the first time the presence of a dysfunctional intestinal barrier after ABD (Antón et al., 2018).

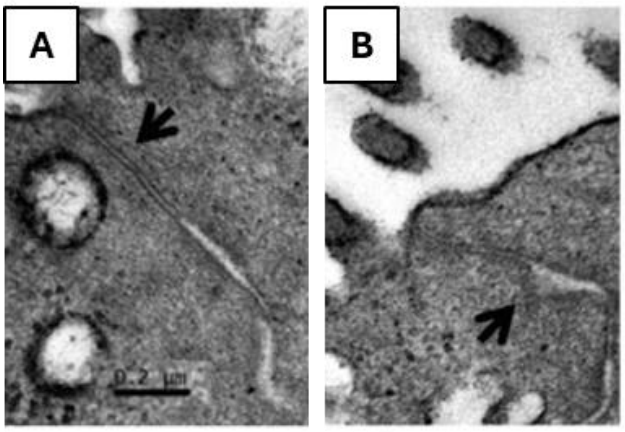



**Figure 3. Transmission electron microscopy images of colonic TJs** from (A) control and (B) ethanol-treated animals (*modified from Antón et al., 2018*). Alcohol binge drinking reduces the expression of TJs among endothelial cells, resulting in an increase in the intercellular distance of the TJ and a compromised intestinal permeability.

Additionally, ABD can induce local intestinal inflammation, which can further exacerbate barrier dysfunction, and alter the permeability of it via paracellular (decreasing the TJs allowing the passage of hydrosoluble molecules) and via transcellular (increasing pro-apoptotic molecules such as caspase 9). This results in the disruption of the intestinal barrier structure and functionality, thereby facilitating the passage of bacteria and their toxins, such as LPS, from the gut to other organs via the bloodstream (Bishehsari et al., 2017). LPS or other toxins are initially identified by innate immune receptors, such as TLR4, which then induce local inflammatory mediators, such as IL-6 and TNF-α. These can activate T and B lymphocytes to produce Immunoglobulin A (IgA) and M (IgM), thereby activating the adaptive immune system. This overexpression of the immune system may result in a disruption of the intestinal barrier, allowing immune cells or toxins to cross into the bloodstream, contributing to systemic inflammation (Antón et al., 2018; Keita & Söderholm, 2010).

### 3.2.3 Peripheral inflammation

ABD modifies the environment of the intestinal bacteria by producing a strong local inflammation in the intestine and the intestinal mucosa, as well as a strong irritation. This results in the activation of the immune system and pro-inflammatory factors, such as cytokines, which in turn have consequences on microbial activity and the functionality of the intestinal barrier, promoting the disruption of the barrier, leaking of proinflammatory products to the circulation, as

explained in the previous section, and finally activates peripheral inflammation (Antón et al., 2018).

As explained before, ABD activates the innate immune system increasing TLR4 expression in peripheral blood mononuclear cells (PBMCs), increasing cytokines levels in plasma, such as TNF-α and IL-1β, and promoting peripheral inflammation (Antón et al., 2017; Orio et al., 2018). On the one hand, these cytokines generated in the periphery can activate HPA axis by vagus nerve and transmit inflammatory signals to the brain. On the other hand, peripheral cytokines may be able to reach the brain parenchyma via circumventricular organs lacking blood-brain barrier (BBB) or by binding to its specific receptors that are expressed on the BBB (W. Banks, 2005; Gonçalves & De Felice, 2021; Pan & Kastin, 2003). Furthermore, as will be discussed subsequently, in certain circumstances the permeability of the BBB is diminished, facilitating the influx of these cytokines and other pro-inflammatory molecules into the brain parenchyma, which in turn gives rise to neuroinflammation.

ABD has also shown to produce bacterial translocation beyond the barrier, observing bacterial growth in the mesenteric lymph nodes and spleen. Although there was not bacterial growth in the liver or blood, there were bacterial components, such as LPS, upregulated in the plasma of animals that underwent binges episodes (Antón et al., 2018). In fact, even a single alcohol binge has shown to increase peripheral endotoxin levels in humans (Bala et al., 2014). This increase in plasma LPS seems to be related with the upregulation of peripheral IgA, which is the main immunoglobulin in the gastrointestinal tract, preventing pathogens from attaching to and penetrating epithelial surfaces (Antón et al., 2018); and IgM, which is the first antibody produced during an immune response in blood and lymphatic tissue (Pasala et al., 2015). However, plasma IgM has shown to be decreased in animals that underwent ABD (Antón et al., 2018) and humans after alcohol abuse (Kazbariene et al., 2007). It is thought that IgA and IgM may have similar functions, but IgM binds to a broader spectrum of bacteria while IgA binds to a more specific set of commensals, cooperating to maximize the homeostasis and immunity (Chen et al., 2020). Increased plasma IgA and IgM levels after IAC may be considered as a peripheral marker of sustained inflammation.

### 3.2.4 Alcohol metabolism in the liver

In recent years, the liver, as the primary metabolic organ for nutrients in animals or humans, has played an indispensable role in the gut-liver-brain axis, acting as a supplement to the gut-brain axis. Indeed, certain liver diseases are intimately associated with neurological disorders via the liver-brain axis, including hepatic encephalopathy and cirrhosis, among others. (X. Sun et al., 2024; Yan et al., 2023).

Liver is specifically susceptible to be damaged by alcohol consumption due to its implication in the alcohol metabolism. The majority of alcohol metabolism occurs in the liver, specifically in hepatocytes, through an oxidative and/or non-oxidative pathway. Following absorption, alcohol reaches the liver via two main routes: diffusion from the upper intestinal tract and via the portal vein. The major pathway of alcohol is via oxidative, were the cytosolic enzyme alcohol dehydrogenase (ADH) is responsible for metabolising it into its toxic metabolite acetaldehyde. Other enzymes, such as cytochrome P450 2E1 (CYP2E1) and catalase, are also involved in this process. Subsequently, the mitochondrial enzyme aldehyde dehydrogenase (ALDH) metabolises this into acetate, which is excreted out of the liver. When high quantities of alcohol are consumed, CYP2E1 is activated and facilitates the generation of acetaldehyde as the first metabolite during alcohol oxidation and the formation of reactive oxygen species (ROS), which are considered as one of the principal causes of liver damage (Figure 4) (Bishehsari et al., 2017; Hyun et al., 2021; Teschke, 2018). Both acetaldehyde and LPS (from the wall of *Gram*-bacteria) activate Kupffer cells promoting the release of ROS and chemokines that recruit bone marrow-derived neutrophils and blood-derived monocytes into the liver (Leevy & Elbeshbeshy, 2005; Osna et al., 2017). One of these chemokines is CXC motif chemokine ligand 1 (CXCL1), which has shown to facilitate the neutrophil infiltration into liver in ethanol-stimulated mice, causing the progression of alcohol-mediated hepatic damage (Y. Jiang et al., 2022; Khan et al., 2023; Roh et al., 2015).

Acetate can be further metabolised outside the liver to acetyl CoA, a key molecule for the synthesis cholesterol, among other molecules. Alternatively, it can enter the bloodstream. Acetate can also reach the brain as a product of the first pass metabolism. Furthermore, alcohol can cross the blood-brain barrier (BBB), where the aforementioned metabolism also occurs. However, in this instance, the initial step is primarily catabolised by the enzyme catalase (Hernández et al., 2016).

The non-oxidative pathway is responsible for a relatively minor proportion of alcohol metabolism. A range of enzymes facilitate the non-oxidative conjugation of alcohol with diverse endogenous metabolites, resulting in the formation of fatty acids, glucuronic acid or sulfates, which have been demonstrated to also cause injury to the liver through the mechanisms of increased lipid accumulation, inflammation, and fibrosis (Hyun et al., 2021).

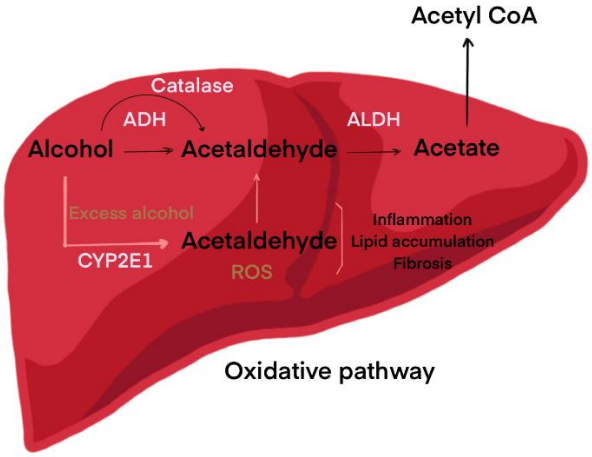



**Figure 4. Alcohol metabolism in the liver** (*Modified from (Hyun et al., 2021)*). Alcohol is metabolised to acetaldehyde by the enzymes ADH in the cytosol, or by the enzyme catalase in the peroxisome. Acetaldehyde is then converted to acetate by ALDH in the mitochondria. Acetate can be further metabolised outside the liver to acetyl CoA. High levels of alcohol activate CYP2E1 and facilitate the formation of reactive oxygen species (ROS), which are thought to be a major cause of liver damage. *CYP2E1*=cytochrome P450 2E1; *ADH*=alcohol dehydrogenase; *ALDH*=aldehyde dehydrogenase.

The liver is the primary organ involved in LPS systemic clearance (De Geest & Mishra, 2022; Kumar et al., 2024), inactivating it and neutralising its inflammatory effects. It is important to note that LPS clearance can be either Apolipoproteins (Apos)-bound, which will be discussed in the Apos section, or non-Apos-bound, being uptake by the liver. Parenchymal cells, namely hepatocytes, and non-parenchymal cells, including Kupffer cells and liver sinusoidal endothelial cells (LSECs), are involved in this process. The clearance of LPS in the liver is thought to be mediated in two phases: a rapid clearance in 5 min and a slow clearance in hours. The first phase is mediated by LSECs (Yao et al., 2016), while the second phase in performed by the hepatocytes and Kupffer cells. Kupffer cells, which are hepatic macrophages, participate in this process by modifying Lipid A structure, which is a small component of LPS which determines its toxicity (Shao et al., 2012). However, following the altered permeability of the intestinal barrier, together with the increase in *Gram*- (LPS-containing) bacteria after ABD, may increase the available LPS to be eliminated so that the clearance process in general may not be fully effective. If high concentrations of LPS pass through the portal vein (the primary source of blood for the liver) and activate TLR4 on hepatic cells, including Kupffer cells, it can result in systemic inflammation. This liver's inability to eliminate excess LPS can also lead to an increase in plasma LPS levels and systemic inflammation (Kumar et al., 2024). This peripheral inflammation has been related to alcoholic hepatitis or alcoholic cirrhosis (Hyun et al., 2021).

### 3.2.5 Blood-brain barrier

It is also possible that components entering the systemic circulation may be modifying the second biological barrier of the BBB. The BBB serves a similar function to that of the intestinal barrier, protecting the brain from toxic substances. As a psychotropic substance, alcohol exerts its effects on the brain by gaining access to this vital organ. Alcohol abuse may induce a leaky of the BBB by its proinflammatory actions at both sides of the barrier, inducing an augmentation of the inflammatory response in a whole (Rodríguez-González et al., 2023; Rubio-Araiz et al., 2017).

The BBB represents a selective barrier along the blood vessels of the CNS. This barrier is of critical importance for the regulation of the microenvironment that is essential for neurons. Effective communication between the nervous systems cells depends on electrical and chemical signals and small changes in these signals can result in failures in cognition, regulation of metabolism, and coordination of peripheral organ functions, among other effects (Keaney & Campbell, 2015; Oller-Salvia et al., 2016).

The BBB is characterized to be a highly capillarised structure composed of different endothelial cells, pericytes and astrocytes end-feet, essential for its correct function. The BBB separates blood from cerebrospinal fluid (CSF), which is a fluid produced by the choroid plexuses, located in the ventricles. CSF plays a role in maintaining the mechanical, biochemical and immunological homeostasis of the CNS. Thus, the BBB protects the CNS from neurotoxic substances, limiting the penetration of toxins and pathogens intro the brain and providing it with essential nutrients and oxygen (Daneman et al., 2010; Obermeier et al., 2016; Oller-Salvia et al., 2016).

Endothelial cells are the anatomical basis of the BBB as in the intestinal epithelium. TJs such as occluding, claudins and ZO-1 regulate the passage of substances from the blood to the CNS back and forth and play an essential role in the formation and maintenance of BBB function, conferring structural integrity and low permeability to the barrier. In the same manner as observed in the intestine, the BBB permits two principal forms of transport: paracellular and transcellular. Paracellular transport enables the passage of water-soluble or small molecules across the BBB mediated by TJs (Tajes et al., 2014). However, in the context of inflammatory processes affecting the BBB, a transcellular route of leakage plays a major role (Erickson & Banks, 2022). Substances that alter the permeability of the BBB will therefore favour the access of pathogens or other substances from the periphery, such as LPS or cytokines, altering the CNS (Abbott et al., 2010; Obermeier et al., 2016).

Ethanol, due to its small, polar molecular structure, can easily cross the BBB. Recent studies from our lab have shown that ABD can alter the integrity of the TJs in animals, reducing the concentration of ZO-1, occluding and laminin and increasing the space between endothelial cells that form the BBB, leading to an enhancement in their permeability (Rodríguez-González et al., 2023). Thus, ABD-derived peripheral inflammation affected BBB functionality by decreasing expression of tight junctions in the PFC, indicative of openness. Chronic alcohol consumption can also decrease ZO-1 in postmortem human alcoholic brain (Rubio-Araiz et al., 2017) and rats (Carrino et al., 2021). It has been suggested that the effects of alcohol in the disruption of the BBB could be associated to the neuroimmune activation, which could favour addictive behaviours in animals (F. T. Crews et al., 2011; Montesinos, Pascual, et al., 2016; Muneer et al., 2012).

It is also conceivable that ethanol may facilitate the transmission of signals from components within the bloodstream to the brain, in addition to exerting direct effects on the brain via ethanol itself. In fact, an increase in plasma cytokine is known to induce brain cytokine responses since they have linked to brain HMGB1/TLR4 activity altered by alcohol (Antón et al., 2017; Zou & Crews, 2014). This would suggest that the peripheral inflammatory response itself may influence the integrity of the BBB. In addition, it is evidenced that bacteria and their associated products have also the capacity to disrupt the BBB. In fact, LPS, that can be increased in plasma after ABD, can alter the expression of TJs (W. A. Banks et al., 2015; Peng et al., 2021) and cases of sepsis have resulted in an increase in BBB permeability (Danielski et al., 2018).

### 3.2.6 Neuroinflammation

A significant process in the neurobiological consequences of alcohol abuse is neuroinflammation. Neuroinflammation is defined as a homeostatic response of the CNS in the presence of damage with the synthesis of neuroimmune system cells, especially microglia. Neuroinflammation and its effects initially serve a protective function to combat an infection or damage to the neuronal environment. This is essential to the repair of the tissue and the restoration of homeostasis, and it is considered neuroprotective when the duration of this inflammatory activity is brief. However, when neuroinflammatory processes are not resolved and they persist over time, they become chronic and detrimental to the CNS. Indeed, chronic neuroinflammation can result in neurodegenerative diseases, such as Alzheimer's disease (AD) or Parkinson’s, and neuropsychiatric diseases, such as depression, due to oxidative stress (García Bueno et al., 2016; Orio et al., 2019; Shastri et al., 2013).

Alcohol-induced neuroinflammation is strongly related with the gut-liver-brain axis disruption (Figure 5). Since ABD alters the permeability of the intestinal barrier and promotes an

increase in the *Gram*- bacteria, higher amounts of LPS can reach the bloodstream and the liver. There, LPS promotes the release of proinflammatory cytokines into the bloodstream, which can subsequently reach the BBB. In fact, the alcohol-induced neuroinflammatory effects are enhanced by external administration of LPS in mice (Qin et al., 2008). Despite the presence of the BBB, the CNS responds to the peripheral inflammatory stimulus by producing neuroinflammation as a local response, and by activating the HPA axis (Obermeier et al., 2016). In addition, alcohol molecule can easily cross the BBB due to its polar nature to exerts its inflammatory function. The immune system is the primary line of host defence against pathogens in the brain. As previously discussed in the section 3.2.2, the activation of TLR4 results in the release of pro-inflammatory mediators, which can lead to the development of astrogliosis, microgliosis and leukocyte infiltration. This pro-inflammatory environment can subsequently induce neuronal damage and neuroinflammation, as well as behavioural effects that will be discussed in further detail below.

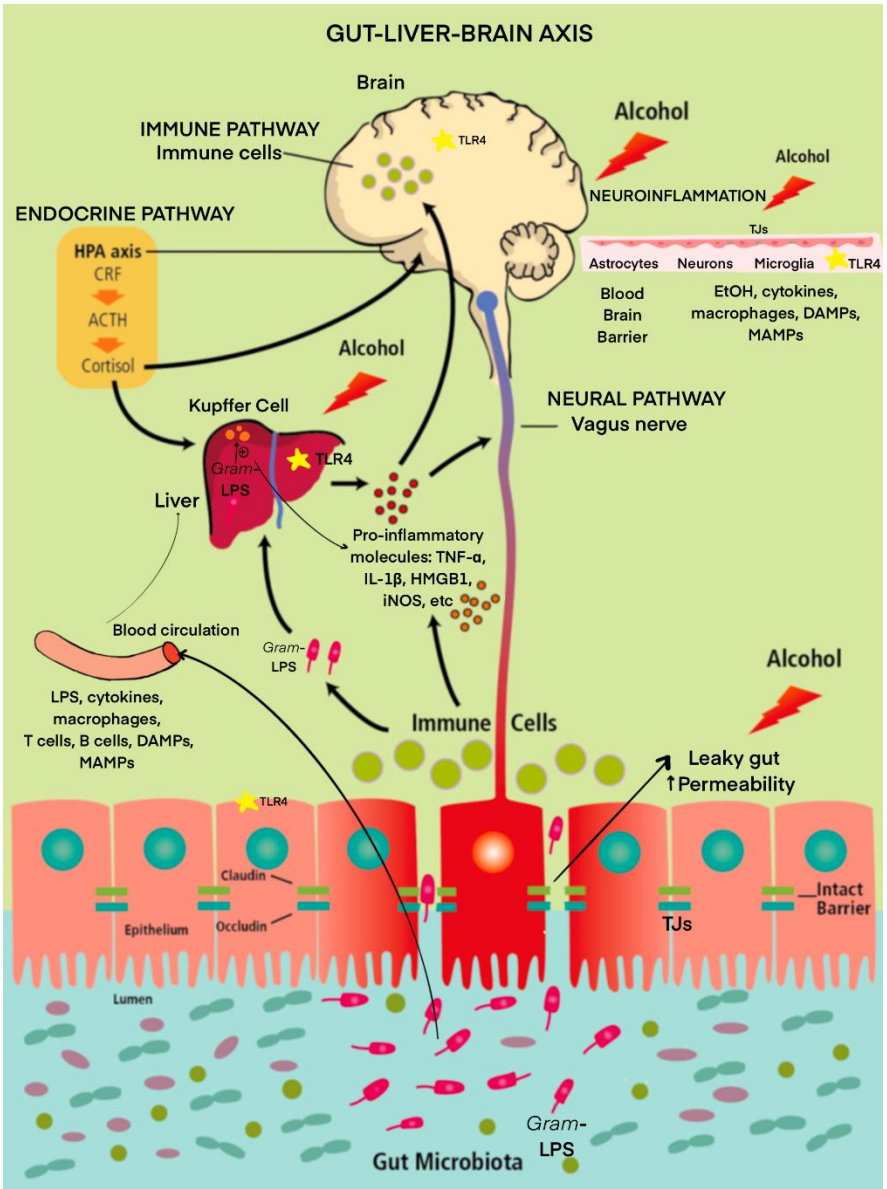


**Figure 5. Gut-liver-brain axis. Alcohol-induced neuroinflammation** (*Modified from Hillemacher et al., 2018*). Alcohol abuse disrupts the intestinal barrier, increases *Gram-* bacteria (LPS-containing) and exerts a local inflammation in the intestine. As a result, LPS and pro-inflammatory molecules, such as TNF-α and IL-1β, are released into the bloodstream, reaching the liver and producing more inflammatory mediators. They can cross the blood-brain barrier, activating astrocytes and microglia, and can cause astrogliosis, microgliosis and leukocyte infiltration. Alcohol can also diffuse directly into the brain by activating the TLR4 pathway in neurons and glial cells. This neuroinflammation can contribute to the production of neuronal damage and has behavioural consequences. *LPS*=lipopolysaccharide*; DAMPs*=cell damage-associated molecular patterns; *MAMPs*=microbe-associated molecules; *EtOH*=ethanol; *IL-1β*= interleukin 1 beta; *TNF-α*=tumour necrosis factor alpha; *iNOS*=inducible; *HMGB1*=high mobility group box 1 protein.

To resume, alcohol abuse can activate the innate immune system via TLR4 and trigger a cascade of inflammatory molecules promoting neuroinflammation in the following ways: (1) directly, alcohol can cross the BBB and promote the synthesis of proinflammatory mediators by a TLR4 direct agonism in both glia and neurons; (2) indirectly, with the upregulation of plasma PAMPS, such as LPS, which can be specifically recognized by TLR4; (3) the overproduction of DAMPS such as HMGB1, which magnifies TLR4 activation; and (4) by cytokines produced in the periphery, specifically at the hepatic and intestinal level, which can either cross the BBB or produce neuroinflammation by signal transduction through the endothelial cells that form the BBB.

### 3.2.7 Behavioural alterations related to alcohol consumption and/or neuroinflammation

The microbiota-gut-brain axis is known to be involved in disorders related to depressive behaviour, anxiety, pathologies related to neurodevelopment, AD, etc. But it could also play a role in addictions and in particular in alcohol abuse and AUD.

As previously outlined, alcohol abuse has the potential to disrupt the microbiota-gut-liver-brain axis. As a result of the disruption to this axis, inflammatory effects are observed in the periphery and in the brain, which may lead to neuronal damage. This constitutes a robust biological basis underlying alterations in animal behaviour during abstinence, such as depressive-like behaviour, anxiety-like behaviour, or anhedonia (Antón et al., 2017; Montesinos, Pascual, et al., 2016; Sayd et al., 2015), although the different patterns of alcohol consumption must be considered to study its effects.

The aforementioned alteration in gut microbiota induced by alcohol has been demonstrated to have behavioural consequences, including an increase in craving, anxiety, depression and other symptoms that are characteristic of alcohol dependence (Hillemacher et al., 2018; Leclercq et al., 2014). Nevertheless, the evidence suggests that alcohol consumption does

not invariably result in gut dysbiosis. This indicates that additional factors may be involved, such conditions including cirrhosis or diabetes (de Timary et al., 2015; E. A. Mutlu et al., 2012), or the pattern of alcohol abuse.

It has been demonstrated that, as a consequence of ABD, plasma LPS levels are increased not only in animals (Antón et al., 2017) but also in humans (Orio et al., 2018), promoting neuroinflammation and having implications in behavioural alterations. The behavioural effects during abstinence after alcohol abuse have been extensively studied, regarding mood alterations, such as depression-like behaviour or anxiety. AUD has a high psychiatric comorbidity, especially with depression. In fact, patients with depression and/or anxiety are more likely to experience AUD than the general population and vice versa (Boschloo et al., 2011; Fergusson et al., 2009). As previously outlined, the TLR4 signalling cascade induces the expression of TNF-α and IL-1β, which are cytokines that have been linked to depressive-like behaviour even in healthy populations (F. T. Crews et al., 2017; Figueroa-Hall et al., 2020; García Bueno et al., 2016; Reichenberg et al., 2001). This is in line with some studies that precede this Thesis, where a reduction in the activation of this cascade of proinflammatory cytokines (by pretreatment with oleoylethanolamide) has led to a decrease in the expression of depressive-like behaviour in animals after ABD (Antón et al., 2017) and a decrease in anhedonia after LPS administration (Sayd et al., 2015).

Anxiety-like behaviours have also been observed in animals that underwent ABD (Evans et al., 2020; Gilpin et al., 2012) or intermittent alcohol consumption (Montesinos, Pascual, et al., 2016). As with depression, it has been observed that the induction of an anti-inflammatory state may have some capacity to combat the increased anxiety induced by ABD during abstinence in male animals exposed to binges (Antón et al., 2017).

It is important to note that the behavioural alterations caused by alcohol abuse and its consequent neuroinflammation extend beyond the emotional level, also affecting cognitive and motor abilities. Moreover, there is evidence indicating a link between adolescent alcohol consumption and subsequent cognitive impairment in adulthood (Alfonso-Loeches & Guerri, 2011; Guerri & Pascual, 2010). This includes alterations in attention, verbal learning, visuospatial processing, memory and motor alterations. Young alcohol binge drinkers have shown to perform more poorly on verbal learning and short-delay memory tasks than non-binge drinkers (Spear, 2018) and also young women drinkers had poorer scores on episodic memory or cognitive flexibility that correlated with elevated levels of inflammatory markers in plasma (Orio et al., 2018). In recent studies in our lab, we observed that men and women patients exhibited lower overall scores on the Test of Detection of Cognitive Impairment in Alcoholism (TEDCA, Jurado-

Barba R et al., 2017) than the general population, indicative of alcohol-related cognitive impairment (Escudero et al., 2023, 2024). Higher plasma LPS levels were also found in these AUD patients, indicative of peripheral inflammation (Escudero et al., 2024). Memory impairments have also been reported with other patterns of alcohol consumption in animal studies: with chronic alcohol treatment (King et al., 2020), correlating positively with the presence of IL-6 in the hippocampus; with intermittent alcohol exposure in spatial (Schulteis et al., 2008) or recognition memory (Beaudet et al., 2016; Marco et al., 2017); and with ABD, which promoted spatial and recognition memory deficits together with neuronal cell death throughout the hippocampal cortical circuits of the brain (Cippitelli et al., 2010), although other authors did not find impairments in spatial memory (Monleón et al., 2022). Regarding motor alterations, recent studies from our laboratory have also demonstrated motor impairment in animals that have been chronically exposed to alcohol, exhibiting disinhibited-like behaviour that correlated with a strong activation of the innate immune system and inflammation (Moya et al., 2022).

## 3.3 Gender/sexual differences in alcohol-induced effects

Gender and sex are understudied variables in most studies on animal/human behaviour and alcohol. The majority of existing research on the gut-liver-brain axis has been focused on male subjects, with a notable absence of comparative studies. Traditionally, preclinical studies have shown a disparity in the extent to which females and males have been studied, with a greater focus on the latter. This is evidenced by the extensive literature on male behaviour after different pharmacological treatments and very scarce literature in female animals. In recent years, however, the necessity for further study of the female subject has become increasingly apparent, given the lack of knowledge regarding the impact in behavioural alterations during abstinence in this sex, and some authors are currently conducting behavioural research comparatively on both sexes (Healey et al., 2022; Rivera-Irizarry et al., 2023; Sanz-Martos et al., 2023).

For example, some studies have examined sex differences in alcohol metabolism. Thus, women appear to have less first-pass metabolism than men, with decreased gastric metabolism in women due to significantly less active gastric ADH, the main reason for sex differences in serum alcohol levels (Baraona et al., 2001; Kezer et al., 2021). Women also have lower body water volume than men, leading to higher BELs (A. M. White, 2020).

The existing literature on gender differences in human in alcohol-induced cognitive effects is inconsistent. Heavy drinking and alcohol dependent women are more likely to have comorbid psychiatric disorders than men (Erol & Karpyak, 2015). Moreover, there are

inconsistent results regarding gender differences in alcohol-induced cognitive effects and toxicity. Some studies have shown that women seem to have a particular susceptibility to the inflammatory and neuropsychological effects of alcohol (Alfonso-Loeches et al., 2013; Orio et al., 2018; Fama et al., 2020; Pascual et al., 2017; Vandegrift et al., 2017) while others have shown that female rat brains appear to be more resistant to oxidative damage due to the antioxidant effect of 17β-estradiol (Jung & Metzger, 2016). Fluctuations in estradiol throughout the menstrual cycle have been linked to various mental health concerns in adult women, including alcohol use (Handy et al., 2022). In this sense, some studies in our group have shown a strong association between ABD-induced peripheral inflammation and neuropsychological performance only in women (Orio et al., 2018). Specifically, a significant negative correlation was found between plasma LPS levels and cognitive episodic memory score in female drinkers in a binge drinking model (Orio et al., 2018).

In spite of these studies, mechanistical studies trying to understand the differences between sexes in response to ABD, with special focus in the gut-brain axis, neuroinflammation and neurobehavioral responses associated to alcohol abstinence are firmly needed.

## 3.4 Can bacterial products reach the brain after ABD?

As explained in previous sections, ABD has been demonstrated to alter the microbiota-gut-liver-brain axis, leading to an increase in proinflammatory molecules in the brain, by different overlapping mechanisms (see below) which are responsible of the alcohol-induced neuroinflammation. It is currently unclear whether, as an additional mechanism, small bacterial components could entry the brain, directly enhancing the neuroinflammatory response.

Indeed, one topic that has been the subject of considerable debate is the manner in which peripheral LPS can stimulate the activation of the innate immune response in the brain (A. K. Singh & Jiang, 2004). Structures lacking the BBB, such as the circumventricular organs, and other blood-brain interfaces, such as the choroid plexus and the meninges, rapidly respond to pro-inflammatory stimuli by LPS (Vargas-Caraveo et al., 2020). The direct infiltration of LPS into brain tissue has been questioned (W. A. Banks & Robinson, 2010), although in pathological conditions, 16S rRNA from bacteria has been identified in post-mortem human brains of patients diagnosed with AD. This evidence supports the hypothesis that bacteria can infiltrate the brain under certain pathological conditions (Emery et al., 2017). Whether alcohol abuse induces the infiltration of LPS into the brain remains largely unknown.

Several mechanisms, not exclusive between them, have been proposed to explain the influence of peripheral inflammation in neuroinflammation, including neural, endocrine and immune routes (see Figure 5):

The **first route**, by means of cytokine signalling through the vagus nerve. Once LPS reach the liver to be cleared, it promotes an inflammatory response from macrophages with the activation of the innate immune system via TLR4, producing the release to periphery of cytokines, such as TNF-α and IL-1β, that can exert a localised effect on the vagus nerve. Once the vagus nerve is activated by peripheral inflammation, it could transmit signals directly to the brain (FT. Crews et al., 2017). The **second pathway** is constituted by activation of transporting mechanisms within the BBB, including vascular prostaglandins. The release of cytokines as a result of LPS effects can stimulate the production of prostaglandins, including prostaglandin E2 (PGE2), through the activation of the ciclooxigenase-2 (COX-2) enzyme, promoting a pro-inflammatory state that facilitates BBB permeability (W. A. Banks et al., 2015). The **third pathway** is attributable to the enhanced permeability of the BBB, which can be attributed to either the direct impact of alcohol and/or alcohol-induced peripheral inflammatory signals (LPS, cytokines, etc.) on TJs (W. A. Banks et al., 2015; Rodríguez-González et al., 2023). Peripheral cytokines can cross the BBB regions where the barrier is leaky and lead to a central immune response. A **fourth pathway** has been proposed through leukocyte infiltration. Somes authors have found vascular leukocyte adhesion factors, such as vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1) increased after LPS treatment, suggesting that inflammatory cells have adhered and transmigrated to the vessels (Haileselassie et al., 2020).

In any case, it is unclear whether, in conditions where the BBB is disrupted, such as in alcohol abuse, the LPS could be able to access brain areas related to alcohol-inducing neuroinflammation. Interestingly, there is a preliminary study showing that the small components of Escherichia Coli LPS, such as Lipid A and Core (see section 3.1.1.1), may infiltrate the brain under physiological conditions when they bound to apolipoproteins (Vargas-Caraveo et al., 2017), which are the main protein part of lipoproteins. Specifically, authors observed LipidA-apolipoprotein aggregates in tanycytes-like cells (interface between blood and CSF) and ependymal cells in circumventricular organs, but they also observed positive staining in brain endothelial cells, for example, in the hippocampal commissure, and even in astrocytes of the medulla oblongata. Astrocytes and tanycytes in the circumventricular organs of the brain are known to be crucial for initiating the LPS-induced inflammatory responses via TLR4 (Miyata, 2022). Thus, we aimed to understand whether under alcohol intoxication binges, where the BBB is disrupted, small components of LPS may directly access the brain in specific areas altered by alcohol consumption.

In addition to the study of Vargas-Caraveo et al., 2017, another study published during the course of this doctoral thesis identified a link between high-density lipoprotein (HDL) molecules and the transportation of LPS to the brain (Radford-Smith et al., 2023). These Apos have been also involved in mechanism of LPS detoxification in the liver (Phillips, 2013). Thus, we aimed to study the possible relationship between different Apos and LPS components in the brain under specific alcohol intoxication conditions.

## 3.5 Apolipoproteins

Apolipoproteins are fundamental proteins that form part of lipoproteins, which are responsible for the transport of lipids within the body. In addition to their established role in lipid metabolism, Apos have recently emerged as a topic of interest in the field of neuroscience and inflammatory research due to their involvement in various biological processes, such as neuropsychiatric pathologies or LPS transport through the bloodstream. Their emerging roles include the potential ability to interact with LPS, the endotoxin component of the *Gram-* bacteria wall, that can induce systemic inflammation and tissue damage. This link suggests that Apos could play a key role in the detoxification of LPS, acting as mediators in the modulation of inflammatory responses and opening new perspectives in the study of their impact on human health.

### 3.5.1 Types of lipoproteins and cholesterol transport

Lipoproteins are water-soluble structures consisting of a neutral lipid core encased within a phospholipid layer that contains cholesterol and one or more Apos, the latter serving as scaffold. These Apos serve several functions, including acting as ligands for cell membrane receptors, as enzyme cofactors for enzymes implicated in the metabolism of lipoproteins and in facilitating the binding with lipopolysaccharide-binding proteins. They also transport lipids in polar water-based solutions such as blood, CSF and lymph (Brandenburg et al., 2002; Feingold, 2021).

Plasma lipoproteins can be classified according to their density, based on their relative contents of protein and lipid, in chylomicrons, very low-density lipoprotein (VLDL), Intermediate-Density Lipoproteins (IDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL).

Plasma lipoproteins are also divided based on the **main Apo content**, which is the protein part. In chylomicrons, the main Apo is ApoB48, although ApoC, ApoE or different types of ApoAI can be found. VLDL is mainly composed of ApoB100, the same as IDL and LDL. Plasma HDL is mainly composed of ApoAI (almost 70%), but other Apos can also be found in a considerably lesser degree, such as ApoE (≤10%), ApoC (10-15%), and ApoM (5-10%) (Figure 6) (Dominiczak & Caslake, 2011; Feingold, 2021; Phillips, 2013; Segrest et al., 2001). Some characteristics are described in the sext section.

Lipoproteins play a key role in the absorption and can be further classified according to the type of **cholesterol transport**: LDL transports the cholesterol from the liver to peripheral tissues and HDL from the tissues back to the liver (known as reverse cholesterol transport) (Figure 6). A further function is the transportation of toxic compounds, such as bacterial toxins. For example, lipoproteins can bind LPS from *Gram*- bacteria or lipoteichoic acid (LTA) from *Gram*+ bacteria, thereby reducing their toxic effects (Feingold, 2021).

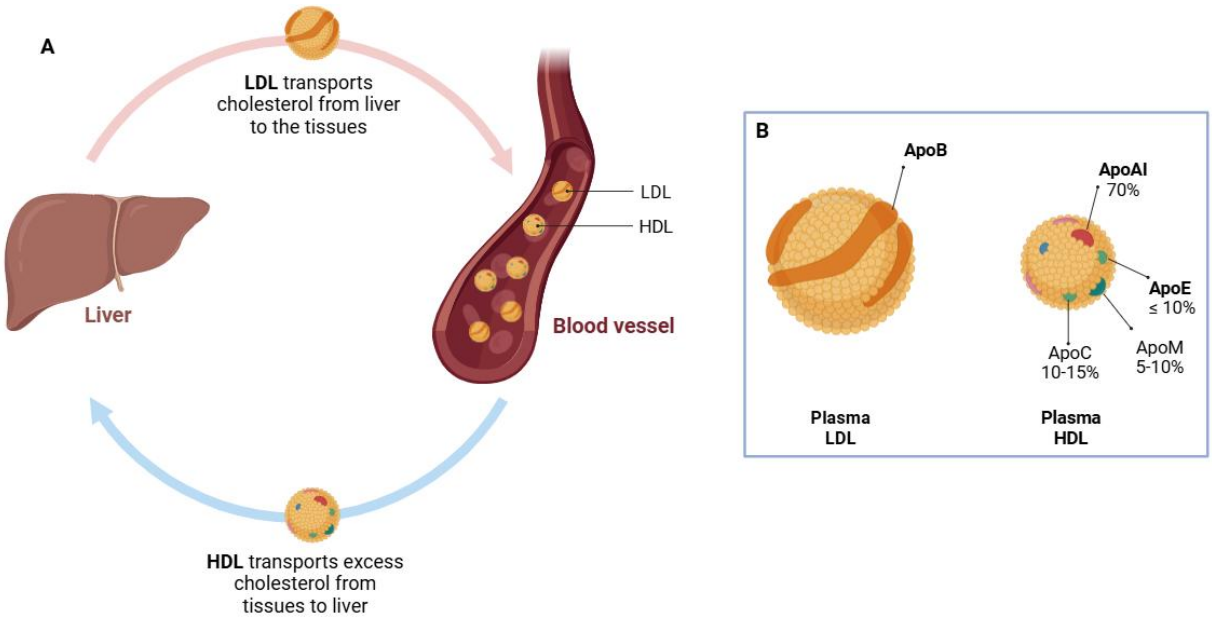



**Figure 6. The process of cholesterol transport and the main content of lipoproteins involved in this process (***Modified from Biorender***). A** LDL transports lipids from the liver to cells while HDL transports excess cholesterol back to the liver. **B** The protein component of lipoproteins is constituted of apolipoproteins. LDL is mainly composed of ApoB. HDL is mainly composed of ApoAI (70%) but other apolipoproteins can be found (ApoE, ApoM, ApoC, etc). *LDL*=low-density lipoprotein; *HDL*=high-density lipoprotein; *ApoB*=apolipoprotein B; *ApoAI*=apolipoprotein AI; *ApoE*=apolipoprotein E; *ApoM*=apolipoprotein M; *ApoC*=apolipoprotein C.

The effects of alcohol consumption on these lipoproteins or its metabolism have been studied. However, this research seems to be related with the development of coronary heart disease. It is to note that some factors, such as the gender, the diet, genetic factors or the drinking pattern, are variable and can modulate the effects of ethanol on plasma lipids (Hannuksela et al., 2002, 2004).

However, alcohol intake has shown to alter some lipoproteins composition. For example, chronic alcohol consumption can increase the quantity of cholesterol and phospholipids in HDL, and it can be associated with an enrichment in polyunsaturated fatty acids, which may impact the antiatherogenic function of HDL particles (Perret et al., 2002). Other studies revealed that HDL particles isolated from individuals with a history of heavy alcohol consumption exhibited reduced efficacy in removing cholesterol from macrophages when compared to HDL particles from control subjects (M. Rao et al., 2000). Ethanol may interfere with the binding of HDL to the scavenger receptor class B type I (SR-BI), maybe affecting the membrane lipid environment or it may directly influence the binding of HDL to the receptor, leading to an increase in plasma HDL concentration (Hannuksela et al., 2002). In humans, moderate alcohol consumption has been associated with increases in plasma HDL levels (Hannuksela et al., 2004) in a dose-dependent fashion and turnover of ApoAI (De Oliveira E Silva et al., 2000), although recent studies suggest that long-term alcohol consumption may decrease HDL serum levels in women (Cho, 2022). Recently, the ratio ApoB/ApoAI has also been studied as a predictor in cardiovascular risk and alcohol intake could modify it (Fu et al., 2024). Reports about the effect of IAC on plasma lipoproteins are very scarce in the field, with some reporting increases in plasma HDL and decreases in LDL profiles in heavy drinkers (Wakabayashi, 2013).

Acute alcohol consumption can also induce an increase in the triglyceride synthesis and production of VLDL particles in the liver (Klop et al., 2013), as well as chylomicrons, as they are large triglyceride-rich particles and alcohol decrease the clearance of chylomicrons in the intestine. The degree of hypertriglyceridemia seems to be related to the stage of alcoholic liver injury (Hannuksela et al., 2002).

The field of sexual differences remains relatively under-explored in this area as well. However, biological sex is one of the most important factors influencing plasma lipid and lipoprotein profiles and plays a key role in the development of cardiovascular disease (Conlon et al., 2023). For example, women secrete a lower number of VLDL particles and higher number of HDL particles than men and body mass index significantly influence ApoAI production and clearance in men, whereas it has no such effect in women (Conlon et al., 2023; X. Wang et al., 2011). The current understanding of the effects of Apos on alcohol consumption is largely based

on their impact on cardiovascular function. However, there is a significant knowledge gap regarding their influence on alcohol-induced inflammation and behavioural alterations. ApoE serves as a notable example of how interactions among genes, gender, and environmental factors can influence circulating plasma lipid levels (Russo et al., 2015). Interactions between the ApoE gene, diet, and alcohol consumption have been observed in men but not in women. Additionally, some studies showed that the ApoE gene affects men and women differently in terms of heart disease risk (Corella et al., 2001; Ordovas, 2007). The complexity of these studies is compounded by the multitude of variables and the diversity of apos that must be considered. Furthermore, alcohol consumption represents an example of an epigenetic or environmental factor, given that it is also a foodstuff that can modify the expression of lipoproteins.

### 3.5.2 Apolipoproteins: types, distribution, functions and receptors

Apolipoproteins play an important role in the body due to their transport function of lipid throughout the aqueous fluids of the body. This occurs because they possess an amphipathic α-helices involved in their lipid-binding capability to be inserted into the cell membranes (Corraliza-Gomez et al., 2019; Elliott et al., 2010). A detailed description of the main Apos studied in this Thesis is provided below.

#### Apolipoprotein AI

ApoAI is a soluble Apo which is the major structural component of HDL in plasma. Peripheral ApoAI is mainly synthesized in the liver and intestinal cells (Meilhac et al., 2020) and as main part of HDL, works to remove excess cholesterol from cells and sends LPS to the liver for its elimination (Berbée et al., 2005; Corraliza-Gomez et al., 2019). However, ApoAI is also naturally present in the brain, but it is supposed to not be secreted there. HDL-particles are up-taken through SR-BI, which is the main lipoprotein receptor for ApoAI and is expressed in endothelial (Balazs et al., 2004) and glial cells (Hottman et al., 2014). ApoAI crosses the BBB back and forth from the circulation to the CSF through endocytosis process to help redistribute cholesterol in the brain (Balazs et al., 2004; Cho, 2022; Fung et al., 2017; Mahley, 2016; Zhou et al., 2019). It is not clear the role of ApoAI in the CNS, but it seems probable that ApoAI plays an analogous role than in peripheral cholesterol transport.

#### Apolipoprotein E

ApoE is a 299 amino acid glycosylated protein of 34-kDa. In plasma, it is present in HDL molecules (≤10%), where it is less abundant than ApoAI (70%) (Mahley, 2016). Peripheral ApoE is mainly synthesized in the liver, and it is involved in the lipid metabolism redistributing

cholesterol and phospholipids for membrane repair and remodelling. ApoE, in contrast to ApoAI, is also secreted in the brain mainly by the astrocytes and, to a lesser extent, by microglia and oligodendrocytes (Elliott et al., 2010; Mahley, 2016; J. Zhang & Liu, 2015). There, ApoE is one of the major soluble Apo (Mahley, 1988) and it is involved regulating the CNS lipid metabolism together with the ApoAI. Apparently, there is no exchange of brain-derived and peripheral free ApoE, although it may cross the BBB when it is bound to HDL (Pedrini et al., 2022). The receptors most involved in cholesterol trafficking in the brain belong to the low-density receptor (LDLR) family and include LDLr and ApoE receptor 2 (ApoER2), which are in charge of the uptake of ApoE-containing lipoprotein particles. They are transmembrane receptors which are highly expressed in astrocytes and neurons (J. Zhang & Liu, 2015).

### Apolipoprotein B

ApoB, as a primary member of insoluble Apo, is the major component of LDL in plasma. There are two principal isoforms: ApoB48, which is found in chylomicrons, and ApoB100, found in LDL and VLDL (K. Singh & Prabhakaran, 2024).In the periphery, ApoB is mainly synthesized in the liver. It plays a role in the absorption of triglycerides, the transport of lipids to peripheral cells, the generation of fatty acids for storage, and lipid metabolism (Mahley, 2016). It is believed that it cannot cross the BBB (Cho, 2022) but some studies have confirmed the presence of ApoB in brain endothelial cells in mice (Lénárt et al., 2015) or bound to αβ plaques in transgenic AD mice (Martins et al., 2009; Takechi et al., 2009). This passage from blood to brain seems to be mediated by LDL receptors (LDLr) by transcytosis, as these receptors are expressed in neurons and glial cells (Corraliza-Gomez et al., 2019; Dehouck et al., 1997; J. Zhang & Liu, 2015). LDLr plays a role in the endocytosis of plasma lipoprotein particles containing apoB100 in the periphery (Jeon & Blacklow, 2005).

The brain is the most cholesterol-rich organ in the body and it is essential to keep brain function well. Moreover, lipid metabolism in the CNS is peculiar (Dietschy & Turley, 2004; Zhang & Liu, 2015). Although lipoproteins in the brain have not been fully studied yet, it is known that the CSF contains lipoproteins which have a density and size similar to plasmatic HDL, known as “HDL-like-particles”, containing mainly ApoE and ApoAI (Camacho et al., 2019; Ito & Michikawa, 2014; Lefterov et al., 2010). The composition of plasma HDL is different from brain HDL as, in the periphery, it is mainly enriched by ApoAI and, in glial cells, is mainly enriched by ApoE (Pedrini et al., 2022; Van Valkenburgh et al., 2021).

There are other plasma apolipoproteins of interest, such as ApoM or ApoJ, that emerge as interesting candidates in the field of AUD and other pathological conditions (Elliott et al., 2010; Escudero et al., 2024; Mousa et al., 2023). However, in this Doctoral Thesis we have focused on ApoAI, ApoE and ApoB, as they are the main components of HDL and LDL.

### 3.5.3 Role of apolipoproteins in neuropsychiatric diseases

Due to their indispensable role in lipid transport and lipoproteins metabolism, apolipoproteins have been extensively studied in the field of cardiovascular biology and atherosclerotic diseases, highlighting the role of the ApoAI, ApoB and ApoE, among others (Mehta & Shapiro, 2022). As previously outline, ABD has inflammatory effects that are observed in the periphery and in the brain, leading to neuronal damage and behavioural consequences. In recent years, the utility of Apos as biomarkers for the diagnosis of different pathologies, like ApoE in specific types of cancer, such as colorectal or brain cancer (He et al., 2022) or in AD (Jackson et al., 2024), has also been emphasized. It is important to note that numerous neuropsychiatric disorders, including AD, have a neuroinflammatory origin that may be exacerbated by alcohol consumption.

Interestingly in the context of this Thesis, Apos have also been identified as novel target molecules associated with inflammation and cognitive processes.

**ApoE**

ApoE has been widely studied in neuropsychiatric diseases. The Ɛ4 allele, which codes for the ApoE4 isoform, has been studied as a biomarker related to cognition. It is known that constitutes an increased risk of AD (Jackson et al., 2024; Shi et al., 2014). In fact, about 60% of clinically diagnosed AD patients carry at least one ApoE4 allele in their genomes (Faraji et al., 2024; Forero et al., 2018). The effects of ApoE4 seem to be related to a decrease in the synaptic function, glucose metabolism and/or mitochondrial functions, as well as neuronal toxicity (Forero et al., 2018). A recent study has linked LBP, ApoE and AD, as they found a significant elevation of plasma LBP levels in AD patients compared to controls, although carrying ApoE4 does not seem to have an effect (Romo et al., 2024). The presence of this isoform also seems to be implicated in the acceleration of the BBB breakdown and degeneration of brain capillary pericytes, contributing to ApoE4-associated cognitive decline (Montagne et al., 2020). ApoE4 mice have also shown alterations in learning and memory and even neural plasticity (Y. Chen et al., 2010; Grootendorst et al., 2005).

**ApoAI**

Other Apos different than ApoE are emerging in the field of neuroinflammation and cognitive processes, such as ApoAI, the main component of HDL in plasma. As cholesterol transport in the brain is strongly dependent on HDL metabolism, this lipoprotein and its main Apo, ApoAI, have emerging roles in neurodegenerative disorders, although HDL in the brain is mainly enriched of ApoE. The role of ApoAI in cognition remains unclear, although some studies have reported that schizophrenic patients presented decreased levels of ApoAI in the brain tissue and CSF, but there was no correlation between CNS and peripheral levels, suggesting independent mechanisms in ApoAI metabolism (J. T. J. Huang et al., 2008). Other studies have recently been identified plasma ApoAI as a biomarker for AD diagnosis (Niu et al., 2024). ApoAI has also been found to be associated with cognition in mice, although it is not clear its role on it. Some authors say it could be protective preserving the cognitive function in animals (Handattu et al., 2009; Lefterov et al., 2010; Lewis et al., 2010) while a recent study from our lab has shown that higher levels of plasma ApoAI correlated with worse cognition on abstinent AUD patients (Escudero et al., 2024).

**ApoB**

ApoB, the major Apo in LDL, has been emerging as a novel biomarker in AD. This Apo seems to alter Tau protein in the early stages of the disease and then enhance amyloid deposition, having implications in cognition (Aumont-Rodrigue et al., 2024). Somes studies suggest that, although there are no detectable levels of LDL in the CSF, increased ApoB can be detected there in AD patients using fluorescence assays. The presence of ApoB in the CSF appears to be related with cognitive decline, predisposing these patients to develop visuospatial cognitive decline (Picard et al., 2022). Some authors have related ApoB overexpression with memory decline and cerebral lipid peroxidation in mice (Löffler et al., 2013) and others with cognitive deficits in depressive patients (Hui et al., 2017). ApoB has also been related with schizophrenia in patients, although some authors have shown both a decrease (Wen & Tan, 2003) and an increase in its expression in this pathology (Mabrouk et al., 2014; Walss-Bass et al., 2019).

### 3.5.4 Role of apolipoproteins in inflammation and alcohol

A multitude of neuropsychiatric disorders have been associated with neuroinflammatory processes. Similarly, addictive behaviours such as alcohol consumption have also been linked to neuroinflammatory mechanisms that contribute to their detrimental effects. Nevertheless, the relationship between Apos, inflammation and alcohol consumption remains poorly understood. In the context of this Doctoral Thesis, given that ABD represents a relatively novel drinking

pattern, there is a scarcity of studies that have investigated its effects related to Apos. The following section presents the current scientific evidence on this topic.

It is established that HDL exerts anti-inflammatory effects in several pathological conditions. For example, it inhibits the formation of arterial plaques, thereby preventing the progression of cardiovascular disease (Fotakis et al., 2019), or regulates the innate immune system by reducing cellular responses to endotoxins, limiting the release of inflammatory mediators (Levine et al., 1993a; Suzuki et al., 2010). In transgenic mice, higher HDL levels have been associated with decreased plasma cytokines levels, suggesting a protective mechanism against endotoxin in vivo (Levine et al., 1993).

### ApoE

ApoE has been studied in depth for its implications in neuroinflammatory processes (Aboud et al., 2014; Duro et al., 2022; Kloske & Wilcock, 2020). As mentioned before, ApoE is practically the Apo most studied, a fact that is also evident in in the context of alcohol abuse and cognitive decline (Downer et al., 2014; Escudero et al., 2023; Slayday et al., 2021), which highlights the necessity for further investigation with other Apos and their impact within this context. However, it has been identified a higher vulnerability to the toxic effects of alcohol in those ApoE4 carriers, even with small consumptions (Slayday et al., 2021). In fact, ApoE4 could enhance ethanol-induced neurotoxicity in mouse cell culture (Li & Cheng, 2018). In a recent study in human in our group, we observed that those AUD patients that were carriers of ApoE4 had worse cognitive performance and higher levels of plasma Reelin, which is a protein that shares receptors with ApoE4, and it is related with inflammation (Escudero et al., 2023).

### ApoAI

There is a notable lack of research examining the relationship between ApoAI and alcohol consumption. As mentioned before, alcohol intake increases plasma HDL concentration (Hannuksela et al., 2002) but little is known about its main Apo, ApoAI. Some authors have shown an upregulation of ApoAI gene expression in hepatoma cells culture after moderate ethanol exposure (Khodja & Samuels, 2020). Recent studies from our laboratory have indicated that ApoAI may serve as a potential biomarker for AUD and associated cognitive impairment, particularly regarding memory. Those AUD patients with higher levels of ApoAI in plasma presented more cognitive impairment (Escudero et al., 2024). Its overexpression in AUD may be related with high inflammation and cognitive deficits. Regarding ABD, ApoAI and HDL have been found decreased in plasma in middle-aged women that underwent ABD (Cho et al., 2022).

**ApoB**

Most studies regarding ApoB, the major component of LDL, are related with cardiovascular diseases and little is known with alcohol abuse. As mentioned before, some studies have reported decreases in plasma LDL profiles in heavy drinkers (Wakabayashi, 2013). In this line, ApoB has been found to be lower in alcoholic patients (Barboriak & Cushman, 1981). Similarly to ApoAI, the effects of alcohol consumption, and ABD in particular, are very scarce in the field.

#### 3.5.4.1 LPS detoxification by apolipoproteins

As described above, small amounts of LPS continuously pass through the intestinal epithelial barrier into the bloodstream under normal physiological conditions and the clearance of LPS takes place in the liver. However, growing evidence suggests that apolipoproteins play roles beyond cholesterol transport, including contributions to immune responses and host defence mechanisms (Hoofnagle & Heinecke, 2009). Thus, lipoproteins have been shown to participate in the detoxification of LPS, acting as key components of the innate immune system (Berbée et al., 2005; Phillips, 2013; Smoak et al., 2010).

The **first step** in LPS detoxification is its binding to HDL. Once LPS is bound to HDL, its inflammatory activity is reduced. Although HDL may protect initially, LPS should be moved to LDL because the `buffering´ capacity of HDL is quickly exceeded (Walley, 2016). In a **second step**, acute phase transfer proteins LBP and PLTP efficiently redistribute LPS from HDL to other lipoprotein subclasses, such as LDL and VLDL, in a time-dependent manner (Levels et al., 2005). Pathogen lipids incorporated into LDL and VLDL are then cleared by the liver via the LDLr (and possibly other lipoprotein receptors such as VLDL receptor (VLDLR), ApoER2 and SR-BI)) and secreted in bile (Topchiy et al., 2016).

On the other hand, since Lipid A, a small toxic part of LPS (explained in section 3.2.1.1), constitutes the insoluble fraction of LPS, it can be incorporated also into micelles, absorbed by chylomicrons and transported to the liver for elimination. This approach would mitigate the toxicity of LPS (Ghoshal et al., 2009; A. Moreira et al., 2012).

Other Apos less studied that seem to be implicated in LPS elimination are ApoE and ApoM. ApoE forms aggregates in the presence of LPS (Martins, 2015; Petruk et al., 2021) and ApoM has demonstrated to neutralize endotoxin activity shuttling LPS to the liver for its detoxification (Mousa et al., 2023).

To the best of our knowledge, Apo-LPS binding was thought to occur exclusively in plasma or liver and to be associated with aspects of endotoxin clearance. Interestingly, some Apos, such as ApoAI, have also been detected in the brain of animals under physiological conditions bound to LPS. This evidence could support the hypothesis that a lipoprotein-mediated transport mechanism may be responsible for the entry of peripheral LPS into the rat brain (Vargas-Caraveo et al., 2017). Other authors have also found an increased in ApoAI in the PFC of animals that underwent a chronic mild stress model, and they also related this increase as a possible transport mechanism of LPS to the brain (Martínez et al., 2021). In this line, a recent study was published during the course of this thesis and showed a significant increase in LPS levels in the brains of animals that received LPS with a pretreatment of intraperitoneal HDL, suggesting that HDL may promote neuroinflammation through direct shuttling of endotoxin to the brain (Radford-Smith et al., 2023).

### 3.5.5 New therapeutic drugs: mimetic peptides

As explained before, HDL may have a role mitigating the activation of the immune system, which is strongly activated by alcohol and/or LPS, and some studies have shown the role of different Apos in the LPS clearance to attenuate the biological response to LPS (Berbée et al., 2005; Henning et al., 2011; Phillips, 2013; Smoak et al., 2010). Considering the growing evidence for the involvement of apolipoproteins in inflammatory and disease processes, together with the use of apolipoprotein-enriched lipoproteins (HDL/LDL), new pharmacological tools have emerged to potentiate the action of specific apolipoproteins. Thus, the properties of HDL have been the subject of extensive investigations as a potential therapeutic agent, due to the ability of HDL to mitigate inflammation and immune system activity (Sviridov et al., 2008), with a particular focus on its efficacy in the prevention of atherosclerosis and coronary heart disease. Intravenous infusion of HDL has also shown to reduce plasma cytokine levels in mice (Levine et al., 1993). However, it is unknown at present, the consequences of its use in a context of alcohol abuse.

Similarly, the enhancement of the biological function of specific apolipoproteins has been the subject of recent research, with a view to establishing it as a new therapeutic strategy (Lynch et al., 2003; R. S. Moreira et al., 2020; Wolkowicz et al., 2021; Wolska et al., 2021). For example, ApoE mimetic peptides has shown to decrease peripheral and brain inflammation in mice after LPS treatments (Lynch et al., 2003) and have antibacterial effects (Petruk et al., 2021; C. Q. Wang et al., 2013). Others can have atheroprotective properties (Wolska et al., 2021). Furthermore, given the role of ApoE in the development of AD and other neurological disorders, there has been

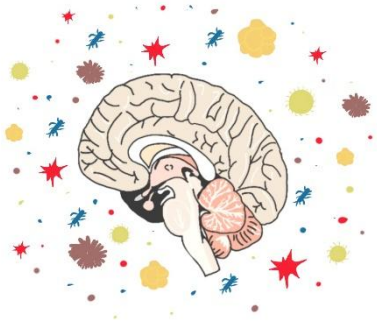
a focus on testing ApoE mimetic peptides in animal models of neurological disease (Chernick et al., 2020).

Regarding ApoAI, some mimetic peptides has been proposed for the study of Parkinson's disease (H. Jiang & Bai, 2022), FAMP peptide for cardiovascular diseases (Uehara et al., 2013) or peptide 5A has shown to enhance remyelination in the CNS (Vanherle et al., 2022). In the field of mimetic peptides, 4F has been shown to have the most significant anti-inflammatory properties (Tao et al., 2024). For example, peptide D-4F has been studied for AD (Handattu et al., 2009; Swaminathan et al., 2020), or its enantiomer, L-4F, has also demonstrated to antagonize the inflammatory effects of LPS and Lipid A in cell culture models (Gupta et al., 2005; Z. Zhang et al., 2009) and septic rats , reducing the expression of inflammatory markers. L-4F is a synthetic ApoAI mimetic peptide containing 18 L-amino acid residues (Ac-DWFKAFYDKVAEKFKEAF-NH2). The presence of 4F may protect ApoAI within lipid complexes from oxidative damage, helping to preserve its anti-inflammatory and anti-atherogenic properties (C. R. White et al., 2019). L-4F also showed to reduce neurovascular and white matter injury by reducing the pro-inflammatory factor HMGB1 and TNF-α in the ischaemic brain of stroke mice (X. Wang et al., 2019).

ApoAI mimetic peptide L-4F appears to play an important anti-inflammatory role in various cardiovascular conditions. Nevertheless, it remains unclear whether its anti-inflammatory effects could mitigate the severe neuroinflammation caused by alcohol abuse and alleviate alcohol-induced behavioural alterations during abstinence. Addressing this question is one of the objectives of this PhD thesis.

In addition to the utilisation of mimetic peptides, another strategy employed is the generation of knock-out (KO) animals, which serve to inhibit the expression of specific Apos, thereby facilitating the investigation of their biological effects. Nevertheless, an analysis of the limited available literature suggests that this technique is not the most frequently employed. For example, ApoAI KO animals have been used as a therapeutic strategy in AD (Contu et al., 2019) or to study diabetes (R. Han et al., 2007) or plasma lipid profile (Y. Wang et al., 2011). There is also evidence of double deletion of ApoE and ApoAI, which ameliorated the amyloid pathology (Fitz et al., 2015). ApoE KO animals have been more studied in the context of atherosclerosis (B. Liu et al., 2023), AD (Avdesh et al., 2011; Henningfield et al., 2022) and even to study the effects of alcohol abuse in atherosclerosis (Furuta et al., 2019).The utilisation of ApoB knock-out animals as a therapeutic strategy is not a common practice, with only a limited number of studies employing this approach. ApoB KO mice have been used to study embryonic lethality and neural tube defects (Farese et al., 1995; L.-S. Huang et al., 1995; Kim & Young, 1998).

# 4. HYPOTHESIS



## HYPOTHESIS

It is established that ABD causes dysbiosis of the intestinal microbiota, increasing the population of *Gram*-negative bacteria, and alters the permeability of the intestinal barrier inducing the translocation of bacterial LPS, a highly proinflammatory molecule, from the intestinal lumen to the systemic circulation. ABD also alters the BBB integrity resulting in an increased exposure of the brain to proinflammatory and toxic compounds and induces neuroinflammation. It is known that the peripheral inflammation induced by alcohol abuse may influence alcohol-induced neuroinflammation and behavioural alterations during abstinence, by activation of indirect mechanisms, such as the release of cytokines or the activation of the vagus nerve. One remaining question is to study whether ABD allows the entry of LPS or its components to the brain, due to a leakage in the BBB, contributing to an augmentation of the neuroinflammatory response induced by the drug.

This PhD thesis therefore sought to investigate whether ABD promotes the passage of peripheral LPS, or its small components such as Lipid A or core, from the peripheral circulation to the brain and to study whether the translocation of them into the brain occurs via binding to specific apolipoproteins, taking into account a sex perspective. It is not clear at present whether a possible binding of LPS to apolipoproteins could be taking place under ABD condition and its functional consequences. Thus, the Apo-LPS aggregates could be involved in the transport or signalling of LPS to the brain or, inversely, they could be homeostatic mechanisms for endotoxin detoxification. In any case, it would have implications for alcohol-induced neuroinflammation: the transport of LPS particles to the brain would have a proinflammatory function whereas the neutralization of LPS by Apos would have anti-inflammatory properties. Whatever the functional consequences, an interesting preliminary research question is to investigate the state of LPS components and apolipoproteins in the brain of male and female animals exposed to alcohol binges, since alcohol abuse disrupt the blood-brain barrier and induces neuroinflammation in brain structures like the PFC. In addition, we aim to investigate the impact of potentiate the effects of specific apolipoproteins on alcohol-induced emotional and cognitive alterations during early abstinence.

Thus, the **general hypothesis** in this PhD Thesis was that ABD induces a translocation of small components of LPS and apolipoproteins from the periphery to brain areas (PFC and/or cerebellum) due to the known BBB breakdown after alcohol abuse in some structures such as the PFC. LPS components will bind to sex-dependent specific apolipoproteins and this process may have consequences in alcohol-induced neuroinflammation and emotional and cognitive alterations during abstinence depending on the sex. Potentiation of specific apolipoproteins

involved in this process by using mimetic peptides will modulate the neuroinflammation and the neurobehavioral alterations observed during ABD abstinence.

The **specific hypotheses** are as follows:

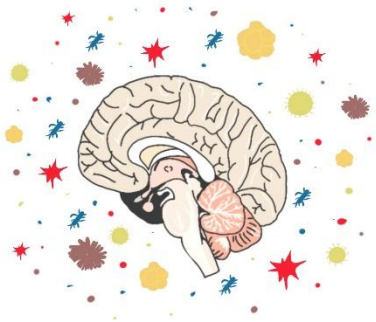
**1.** ABD promotes an increase in the LPS and LBP levels in plasma in male and female animals and also allows the entry of LPS or its small components (Lipid A and Core) into brain structures related to alcohol abuse, such as PFC and cerebellum. It is possible to detect a minimal presence of these components in the brain under physiological conditions in males and females, and higher expression in animals exposed to an ABD protocol.

**2.** ABD induces alterations in the expression of apolipoproteins, such as ApoAI, ApoB and ApoE, specific of sex, both in plasma and in brain areas (PFC/cerebellum) and in their respective receptors (SR-BI, LDLr and ApoER2).

**3.** Animals exposed to ABD episodes show aggregates of LPS components (Lipid A) with apolipoproteins, dependent of sex, in brain structures (PFC and cerebellum), which may be related to neuroinflammation.

**4.** Apos-containing lipoproteins or Apo mimetic pre-treatments that enhance sex-specific apolipoprotein content may have an impact on alcohol-induced neuroinflammation and behaviour, such as the motivational state and anhedonia, anxiety, depressive-like behaviour and/or memory functioning during ABD abstinence.

# 5. OBJECTIVES



The **general objective** of this Doctoral Thesis is to characterise the presence of LPS components, such as Lipid A and Core, in its free form or bound to specific apolipoproteins, in the PFC and the cerebellum of animals exposed to forced alcohol binges and to understand its relationship with neuroinflammation and neurobehavioral alterations during abstinence, with a sex perspective.

The aforementioned objective will be achieved through the following **specific objectives:**

**1.** To explore sexual differences in the effects of an ABD animal model traditionally validated for males, including achieved BELs, plasma corticosterone levels, mortality, affectation of hepatic alcohol enzymes, etc.

**2.** To study alterations of LPS and its binding protein LBP in plasma as well as the presence of small LPS components (Lipid A and core) within the brain (PFC and cerebellum) of male and female animals exposed to ABD preclinical models that have shown validity to induce neuroinflammation and behavioural alterations during early abstinence.

**3.** To study alterations in plasma lipoproteins (HDL/LDL) and apolipoproteins (ApoAI, ApoB, ApoE) and their receptors (SR-BI, LDLr, ApoER2) in plasma and/or brain (PFC and cerebellum) after forced ABD, considering the impact of sex.

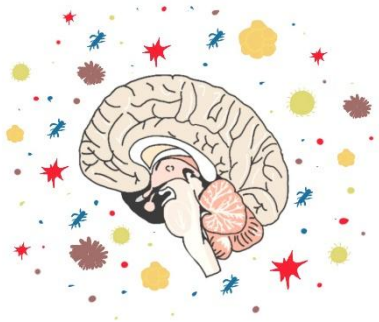
**4.** To study the presence of brain aggregates between specific apolipoproteins, found altered in the previous objective, and small LPS components by western blot in animals of both sexes exposed to intensive alcohol consumption (IAC) and confirm the binding by using co-immunoprecipitation. To explore relationships of the aggregates, if any, with alcohol-induced neuroinflammation in male and females.

**5.** To potentiate the effect of specific Apos that have emerged in previous objectives, by using Apo-enriched lipoproteins and specific Apo mimetic pre-treatments in:

**5.1** The effect of ABD-induced neuroinflammation (by measuring the proinflammatory molecules: TLR4, IL-1β, IL-6, TNF-α, CXCL1 and/or HMGB1).

**5.2** The impact on behavioural alterations during early alcohol abstinence, such as motivational behaviour and anhedonia, anxiety, depressive-like behaviour and spatial and recognition memory.

# 6. MATERIALS AND METHODS



## 6.1 Animals

A hundred and eighty Wistar rats (Envigo©, Barcelona, Spain) weighing 200-225 g were used across all experiments. Female and male animals were housed in different isolated rooms. In any case, upon arrival, animals were housed in groups of 2-3 per cage and maintained at constant conditions of temperature (21 ± 1°C) and humidity (59 ± 10%) under a reverse 12h dark-light cycle (lights on at 8:00 p.m.) with free access to food and water. Animals were habituated to these conditions for one week before the experiments, at which time they were handled gently to acclimate them to the experimenters and gavage procedure.

All procedures were approved and adhered to the guidelines of the Animal Welfare Committee of the Complutense University of Madrid (Ethical approval references: PROEX 312/19 and 122.7-23) following European legislation (2010/63/EU).

## 6.2 Experimental design

Animals received intragastric (i.g.) doses of ethanol or water three times per day for four days using specific cannulae (16-G needle, Fisher Scientific, Waltham, MA, USA), following a standard paradigm of a 4-day forced binge alcohol intoxication protocol previously used by our group (Antón et al., 2017; Rodríguez-González et al., 2021) and by others (Obernier, Bouldin, et al., 2002; Obernier, White, et al., 2002). Animals receive a loading dose of 5g/kg of ethanol 30% and 3g/kg for the subsequent doses. This means that rats receive a maximum dose of 9g/kg/day and they showed characteristic signs of ethanol intoxication, such as ataxia, sedation or loss of reflex, as described by Majchorowicz 1975 (Majchrowicz, 1975) (See Figure 7A). Ethanol solutions were prepared from 96% ethanol stock diluted in water, and body weights were measured daily 2h before the beginning of the i.g. gavage.

In the pharmacological and behavioural studies, we used in females a modification of the ABD protocol previously described, by introducing 2-day period or abstinence (Figure 7B), due to the importance of abstinence effects in the behavioural manifestation of alcohol abuse (Pascual et al., 2017; Sey et al., 2019; Tapia-Rojas et al., 2018). These animals received the same doses of alcohol binges than previous protocols in a 2 days on/2 days off/2 days on protocol. Animals were administered an intraperitoneal (i.p.) pre-treatment of ApoAI mimetic compounds (see section 6.3). All behavioural assessments were conducted after the treatments over the following two weeks, as shown in Figure 7C.

**A**

| TIME | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 |
|---|---|---|---|---|---|
| 7:00 | | EtOH 3g/Kg | EtOH 3g/Kg | EtOH 3g/Kg | EtOH 3g/Kg |
| 15:00 | EtOH 5g/Kg | EtOH 3g/Kg | EtOH 3g/Kg | EtOH 3g/Kg | |
| 23:00 | EtOH 3g/Kg | EtOH 3g/Kg | EtOH 3g/Kg | EtOH 3g/Kg | |

**B**

| TIME | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 | DAY 6 | DAY 7 |
|---|---|---|---|---|---|---|---|
| 7:00 | | PT i.p. EtOH 3g/Kg | PT i.p. EtOH 3g/Kg | | | PT i.p. EtOH 3g/Kg | PT i.p. EtOH 3g/Kg |
| 15:00 | PT i.p. EtOH 5g/Kg | PT i.p. EtOH 3g/Kg | | | PT i.p. EtOH 3g/Kg | PT i.p. EtOH 3g/Kg | |
| 23:00 | PT i.p. EtOH 3g/Kg | PT i.p. EtOH 3g/Kg | | | PT i.p. EtOH 3g/Kg | PT i.p. EtOH 3g/Kg | |

**C**

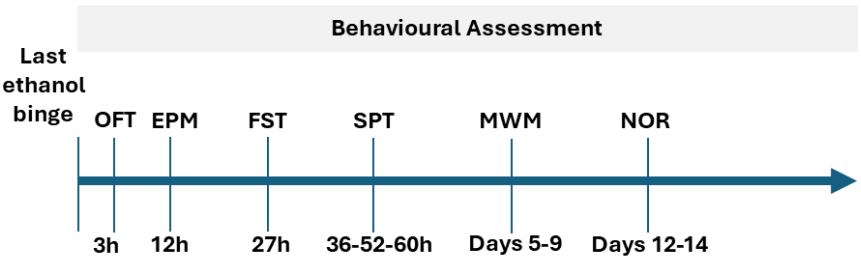



**Figure 7. Experimental design of the study**. **A** Intensive alcohol drinking protocol. Animals received alcohol intragastrically (i.g.) (3 g/Kg) every eight hours over four consecutive days, with a loading dose of 5g/Kg. **B** Modified alcohol binge protocol in a 2-day on/2-day off protocol. Animals received the same doses of ethanol than in protocol A but a period of abstinence was introduced in the middle of the protocol. Animals received a pharmacological treatment (PT) intraperitoneally (i.p.) 15 min before each ethanol binge. **C** Timeline of behavioural assessment during ethanol abstinence. *PT*=pharmacological treatment; *EtOH*=ethanol; *OFT*=open-field test; *EPM*=Elevated Plus Maze; *FST*=Forced Swimming Test; *SPT*=Saccharine Preference Test; *MWM*=Morris Water Maze; *NOR*=Novel Object Recognition.

The introduction of the sex perspective in preclinical studies is fundamental, when possible. Since LipidA-apo aggregates found after ABD differ by sex, we chose females for the pharmacological and behavioural studies. This decision was based on time limitations of this Doctoral Thesis. Additionally, females have been traditionally less studied than males in preclinical studies, a fact that can be observed by looking at the extensive literature on male behaviour, which led us to prioritize the behavioural studies with females versus males.

## 6.3 Pharmacological treatments

### 6.3.1 High density lipoprotein (HDL)

HDL (from human plasma, 361-10. Lee Biosolutions, USA), which major apolipoprotein content is ApoAI, was administered at a dose of 20 mg/Kg (i.p.) solubilized in sterile saline prior to every ethanol binges. This dose of HDL was proven to be pharmacologically active in previous studies (Radford-Smith et al., 2023). Upon arrival, it was thawed in a 37°C water bath and then it was maintained at 4°C during the experiment. Tissue and plasma samples were collected as explained in section 6.5.

### 6.3.2 ApoAI mimetic peptide (4F)

The ApoAI mimetic peptide 4F (also known as APL180) was donated by Dr. GM Anantharamaiah and his group from the University of Alabama (Birmingham, U.S.A.) and synthesized by the solid phase peptide synthesis method as previously described (Datta et al., 2001). Animals received a dose of L-4F peptide (5 mg/Kg, i.p.) (sequence: Ac-DWFKAFYDKVAEKFKEAF-NH2) solubilized in sterile saline 15 min before every ethanol binge. The dose of 4F was proven to be pharmacologically active in previous studies (Bloedon et al., 2008; Geeta Datta, 2011; R. S. Moreira et al., 2014).

## 6.4 Estrous cycle

The female reproductive cycle was controlled during this first experiment by collecting vaginal smears once a day at the same time of the day to reduce variability. Vaginal secretions were collected with a plastic pipettes filled with normal saline (NaCl 0.9%) by introducing the tip gently into the rat vagina, and the vaginal fluid was placed on different glass slides and immediately examined under a light microscope. Estrous cycle phases were determined by observation of cell types in the entire smear (Marcondes et al., 2002) by using a Nikon Japan microscope (Nikon Instruments, Inc., Melville, NY).

## 6.5 Tissue and plasma collection

Following an alternation of the experimental groups, samples were taken three hours after the last ethanol administration. This time-point for sample collection was chosen based on previous results of the group, since most of the neuroinflammatory markers peaked at that time (Antón et al., 2017).

Animals were administered a lethal dose of sodium pentobarbital (320 mg/kg, i.p., Dolethal®, Spain) and blood was collected by cardiac puncture using ethylenediaminetetraacetic acid (EDTA) (molecular weight 452.24 g/mol, pH 7.2) or heparin as an anticoagulant, prior decapitation. Blood samples were centrifuged at 4°C for 15 min at 2,000 × g for plasma fraction collection, which was stored at -80°C until assay. Brains were rapidly isolated from the skull, discarding blood vessels and meninges, and the PFC was excised and frozen at -80°C until assayed.

An additional control group (n=8) with no treatment was included in a pilot study to study the contribution of blood vessels to the results. These animals followed control conditions, but after anaesthesia with sodium pentobarbital they were perfused with sterile physiological saline solution (0.9% NaCl) prior decapitation.

## 6.6 Western blot

As explained by (López-Valencia et al., 2024), brain samples were homogenized by sonication in PBS (pH=7.4) mixed with a protease inhibitor cocktail (Complete, Roche®, Madrid, Spain) at a dilution of 1:3 (w/v), followed by centrifugation at 13,000 rpm at 4°C for 10 min. Protein levels were measured and adjusted by Bradford's method, and homogenates were mixed with Laemmli simple buffer (Biorad®, Alcobendas, Madrid, Spain) containing β-mercaptoethanol (50 µL/mL of Laemmli) to obtain a final concentration of 1 mg/mL. Proteins were separated by an electrophoresis gel, blotted onto nitro-cellulose membranes (Amersham Ibérica®, Madrid, Spain) with a semidry transfer system (Bio-Rad®, Madrid, Spain), incubated with specific primary and secondary antibodies (See Table 1) and revealed by using a chemiluminescence system (ECL™-kit) (Amersham Ibérica®, Madrid, Spain). Autoradiographs were quantified by densitometry (NIH ImageJ® software, National Biosciences, Lincoln, Nebraska USA) and expressed as optical density (O.D.). In all Western blot analyses, the housekeeping β-actin or GAPDH protein was used as a loading control. Every blot contained different samples per group, and 2 blots were run in separate assays. The results represent the

average of two technical replicates. When necessary, a stripping procedure was performed, as indicated in the representative blots of the figures. The samples of western blot were loaded separately for male and female groups, so no direct comparation between sexes was done in these analyses.

In a pilot study, we demonstrate that Lipid A is bound to different proteins, all of which are of interest in our study (López-Valencia et al., 2024). This binding activity was visualized by both western blot and coimmunoprecipitation (co-IP) procedures (next section). The analysis of bound forms between Lipid A and apolipoproteins by western blot was first performed by incubation of samples with the antibody against the specific protein of interest (i.e., ApoAI). Then, a stripping procedure was performed, and the membranes were incubated with the antibody against Lipid A, which shows a band with a similar molecular weight to the protein of interest (note that free Lipid A weighs ~10 kDa), indicative of the [Lipid A-protein] complex (which has also been demonstrated by co-IP procedures). The results of the binding between Lipid A and each apolipoprotein were expressed as the quantification of the bound form normalized by the total amount of the specific protein/apolipoprotein in this brain area (i.e., [LipidA-ApoAI]-bound form were normalized by the total expression of ApoAI (free + bound form; incubation with antibody against ApoAI)).

In the pilot study, to double check the binding (i.e., [LipidA-TLR4], see screening study in the section 7.1.4.1), we first precipitated the conjugate with the antibody against Lipid A, and then the complex was incubated by western blotting with the antibody against TLR4. Then, another set of samples was prepared and we first immunoprecipitated the complex using the antibody against TLR4. The complex was then incubated by western blotting with the antibody against Lipid A.

**Table 1. Specific antibodies used in western blotting to detect proteins of interest**

| Protein | Primary Antibody | Secondary Antibody |
|---|---|---|
| **Ligands** | | |
| ApoB | 1:500 sc-393636 | Mouse (1:2000) |
| ApoAI | 1:4000 Ab20453 | Rabbit (1:3000) |
| ApoE | 1:500 sc-390925 | Mouse (1:2000) |
| Lipid A | 1:500 Acris BP2235 | Goat (1:3000) |
| Core | 1:500 HM6011 | Mouse (1:2000) |
| **Receptors** | | |
| LDLr | 1:500 Ab30532 | Rabbit (1:2000) |
| SRBI | 1:1000 NB400-104 | Rabbit (1:2000) |
| TLR4 | 1:500 BSA 1% sc-293072 | Mouse (1:2000) |
| CD14 | 1:1000 ab203294 | Rabbit (1:2000) |
| **Housekeeping** | | |
| β-actin | 1:10000 A5441 Sigma | Mouse (1:10000) |
| GAPDH | 1:5000 G8795 Sigma | Mouse (1:5000) |

## 6.7 Co-immunoprecipitation

Co-IP is a biochemical method to precipitate a complex using target-specific antibodies. In this study, co-IP was used to study LipidA-Apo binding (protein–protein aggregates). The Lipid A antibody was first used to immunoprecipitate, and then it was immunoblotted using specific ApoAI, ApoB and TLR4 antibodies (ApoE was not used because no colocalization of Lipid A and ApoE was found in western blotting). The signal obtained in the co-IP means that ApoAI, ApoB or TLR4 is immunoprecipitated in Lipid A, confirming the binding of Lipid A to ApoAI, ApoB or TLR4 (Figure 8).

Co-IP was performed based on a previously published protocol (García-Negredo et al., 2014; Morató et al., 2016). Brain samples were mechanically homogenized using 5 mm stainless steel beads in a TissueLyser LT (Qiagen®, Hilden, Germany) with 1 mL of 50 mM Tris buffer (pH=7.4) mixed with a protease inhibitor cocktail for each brain tissue sample. The frequency used was 50 oscillations for 2 min 3 times, followed by centrifugation at 1 000 × g for 10 min at

4°C. The supernatants were collected and centrifuged at 12 000 × g for 30 min at 4°C. Then, the pellets were resuspended in Tris buffer at a dilution of 1:750 (w/v). Protein levels were measured and adjusted by Bradford's method, and homogenates were mixed with Tris buffer to obtain a final concentration of 4 mg/mL.

To obtain concentrated samples from small amounts of tissue and due to the low signal intensity obtained after Co-IP during the first trials, a sample pooling of 2 biological replicates in a group was performed. All samples were centrifuged at 12 000 × g for 30 min at 4°C, and pellets were resuspended in 200 µL of RIPA buffer (R0278, Sigma–Aldrich®, Madrid, Spain) containing protease inhibitor cocktail and incubated for 30 min with constant rotation at 4°C. Samples were centrifuged at 12 000 × g for 30 min at 4°C, and supernatants were collected in Eppendorf tubes containing 10 µL of Lipid A antibody (See Table 2) and incubated overnight with constant rotation at 4°C to allow the formation of immune complexes. Twenty-five microliters of Protein A Agarose resin (P3476, Sigma–Aldrich®, Madrid, Spain) was added to the samples and incubated with constant rotation for 2 h at 4°C to collect the immune complexes. The resin was washed three times by centrifuging at 10 000 × g for 1 min at 4°C. Resins were first resuspended in 200 µL of RIPA buffer and, in the last wash, with Laemmli simple buffer containing β-mercaptoethanol, and they were analyzed by SDS–PAGE and immunoblotted using ApoAI or ApoB antibodies (Table 2).

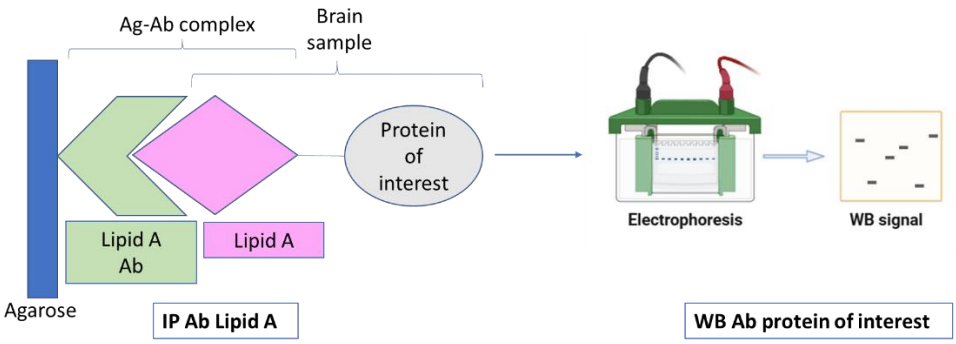



**Figure 8. Schematic representation of the co-IP process as a direct measure of colocalization of Lipid A and proteins of interest**. ApoAI, ApoB and TLR4 are de proteins of interest in these studies. *Ag*=antigen; *Ab*=antibody; *IP*=immunoprecipitation; *WB*=western blotting.

**Table 2. Antibodies used in co-immunoprecipitation to precipitate the protein complex**

| Co-IP Antibody | Primary WB Antibody | Secondary WB Antibody |
|---|---|---|
| **Lipid A**<br>**Acris BP2235** | ApoB 1:500 sc-393636<br>ApoAI 1:4000 Sc-135837<br>TLR4 1:500 BSA 1% sc-293072 | Mouse (1:2000)<br>Rabbit (1:3000)<br>Mouse (1:2000) |
| **TLR4**<br>**sc-293072** | Lipid A 1:500 Acris BP2235 | Goat (1:3000) |

## 6.8 Enzyme-Linked Immunosorbent Assay (ELISA) kits

**Blood ethanol levels (BELs) determination**

Ethanol levels in plasma samples were measured by the commercial Enzychrom TM Ethanol Assay Kit ECET-100 (BioAssay Systems®, Hayward, CA, USA) according to the manufacturer's protocol. The absorbance of each well was measured at 570 nm using a ThermoMax microplate reader (Molecular Devices®, Ramsey, USA).

**Plasma corticosterone levels**

Plasma corticosterone levels were determined by a colorimetric competitive enzyme immunoassay kit (Catalog No. ADI-900-097, Enzo Life Sciences®, Lauren, Switzerland) following the manufacturer's instructions. Standards and plasma samples were assayed in duplicate. Absorbance was measured at 405 nm using a ThermoMax Microplate reader (Molecular Devices®, Ramsey, USA). Calculated values are expressed as nanograms of corticosterone per milliliter (ng/mL).

**Plasma LPS determination**

Plasma LPS levels were determined using a commercially available kit based on enzyme-link immunosorbent assay (ELISA) following the manufacturer's instructions (Hycult Biotech®, Uden, The Netherlands). This test is based on the ability of the endotoxin to cause intravascular coagulation in the American horseshoe crab, Limulus polyphemus. This endotoxin causes an opacity and gelation in Limulus amebocyte lysate (LAL), producing an enzymatic reaction and a yellow colour. LPS was measured at 450 nm (Molecular Devices®, Ramsey, USA). The results were obtained as endotoxin units per mL (EU/mL) and expressed as a percentage of control values.

**Brain LPS determination**

Brain LPS levels were determined using Pierce™ Chromogenic Endotoxin Quant Kit (A39553, Thermo Fisher Scientific Inc., Spain). This kit is a LAL assay for endotoxin quantitation. All the equipment was sterile and pyrogen-free. Brain tissue was homogenized by sonication in 3 volumes of PBS followed by centrifugation at 13,000 rpm at 4°C for 15 min. Supernatants were collected and diluted 1:5. Quantitation of endotoxin was performed following the manufacturer's instructions. Absorbances were measured at 405nm using a ThermoMax microplate reader (ThermoMax microplate reader. Molecular Devices®, Ramsey, USA) and the results expressed as a percentage of control values.

**Determination of apolipoproteins**

ApoAI, B and E in plasma samples were measured using the sandwich-ELISA principle with a commercial assay kit (Catalog No. E-EL-R3029, E-EL-R1218, E-EL-R1230, respectively, Elabscience Biotechnology® Inc., USA) following the manufacturer's instructions. The absorbance was measured at 450 nm using a ThermoMax microplate reader (Molecular Devices®, Ramsey, USA).

**HDL and LDL levels**

HDL and LDL levels were measured in rat plasma using commercially available sandwich enzyme immunoassays (SEB006Ra-96T and SEB107Ra-96T, respectively, Cloud-Clone Corp.®, TX, USA). The final concentrations of HDL and LDL in the samples were determined by comparing the O.D. of the samples to the standard curve by measuring the colour change spectrophotometrically at 450 nm wavelength (ThermoMax microplate reader. Molecular Devices®, Ramsey, USA).

**Determination of proinflammatory cytokines**

TNF alpha and IL-1β were analysed in rat PFC using a commercially available kit based on enzyme-link immunosorbent assay (ELISA) following the manufacturer's instructions (ELR-TNF-α-CL and ELR-IL1β-CL, respectively. RayBiotech, Georgia, EEUU). The stop solution changed the colour from blue to yellow and the intensity of the colour was measured at 450 nm (ThermoMax microplate reader. Molecular Devices®, Ramsey, USA).

**Alcohol metabolism: ADH and ALDH levels**

ADH and ALDH enzymes were analysed in liver samples by a quantitative sandwich enzyme immunoassay (CSB-E11180r, Cusabio®, TX, USA) and a competitive enzyme immunoassay (MBS724302, MyBioSource®, CA, USA), respectively, following the manufacturer's instructions. The intensity of the colour was measured at 450 nm (ThermoMax

microplate reader. Molecular Devices®, Ramsey, USA) but in the ALDH kit it was inversely proportional to the ALDH concentration.

## 6.9 Quantitative polymerase chain reaction (qPCR)

This part of the methodology was carried out during the international stay in collaboration with Prof. Daniel Anthony's group, from the Department of Pharmacology (University of Oxford).

### 6.9.1 RNA isolation

PFC was excised in dry ice from the frozen brain tissue stored at -80°C and then weighted to obtained 15-20 mg of tissue. RNA extraction was carried out using the Qiagen RNEasy® Mini Kit (Qiagen Ltd, Manchester, UK) as per the manufacturer's instructions.

In sterile eppendorfs, the tissue was homogenised with 300 µL of buffer solution previously prepared (RLT buffer with 1% β-mercaptoethanol) using a Kimble® pellet pestle® motor cordless. Once it was homogenised, another 300 µL of buffer solution was added and vortex vigorously.

Each sample were transferred to a labelled Purple Column (QIAshredder Mini Spin Column, Qiagen) followed by centrifugation at 13,000 rpm for 3min at 20°C. The purple columns were discarded, and the filtrates kept. 600 µL of prepared 70% ethanol were added to each sample using the pipette to mix into solution slowly to avoid bubbles, having a total volume of 1,200 µL in the sample. The samples were transferred to the Pink Column (QIAshredder Mini Spin Column, Qiagen) and centrifuged at 10,000 rpm for 20 s at 20°C. The filtrates were discarded as the pink columns now contained RNA.

RNA needed to be washed to remove more impurities with RWI and RPE buffers. 700 µL of RWI were added once to each pink column once followed by centrifugation at 10,000 rpm for 30 s at 20°C, and the flowthroughs were discarded. Then, 500 µL of RPE were added twice and centrifuged at 10,000 rpm for 30 s at 20°C the first time and 10,000 rpm for 2 min at 20°C the second time, discarding the flowthroughs each time.

Pink columns were transferred to new collecting tubes and centrifuged again at 13,300 rpm for 1 min at 20°C. Should be nothing or very little in the new collecting tube. Finally, pink columns were transferred to another new collecting tube and 30 µL of RNase-free water were

added to each. Drop needs to be central on membrane. All of them were centrifuged at 10,000 rpm for 1 min to elute the RNA. The flowthroughs now contain purified RNA and they were immediately stored at -80°C until assayed.

### 6.9.2 cDNA conversion

To verify the RNA purity, a NanoDrop 1000 Machine was used (Thermo Fisher, UK) and was considered suitable for cDNA conversion if the 260/280 nm ratio (indicating the extent of genomic DNA contamination) was >2 and the 260/230 ratio (indicating phenol contamination) was between 1.8 and 2.2.

The concentrations of RNA obtained with the nanodrop were normalized to 100 ng/µL (1000 ng of RNA per 10 µL of sample) with RNase-free water. RNA was then converted to cDNA using the high-capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Fisher Scientific, Warrington, UK), as per the manufacturer's instructions, adding 10 µL of the Master Mix to each sample, getting a final volume of 20 µL. Samples were put in the thermocycler (Biorad® T100 Thermal Cycler, EEUU) at 25ºC for 10 min, at 37ºC for 120 min and at 85ºC for 5 min. The cDNA was stored at -20ºC until use.

### 6.9.3 Quantitative (q)PCR

Real-time or quantitative polymerase chain reaction (real time-PCR or qPCR) allows comparative amplification and quantification of the mRNA of the protein to be determined. This is made possible by a non-specific intercalating fluorophore. Here, we used SYBR® Green Supermix (Biorad®), excited by blue light and emitting green light and samples were run using a LightCycler® 480 instrument (Roche Diagnostics, West Sussex, UK).

25 ng cDNA was used per reaction. Primers were purchased from either Primerdesign, Merck Life Science Ltd (Dorset, UK), or Bio-Rad Laboratories Ltd (Hertfordshire, UK), and used at a working concentration of 300 nM. GAPDH was used as the reference gene. Primer sequences can be found in Table 3. The generation of a melt curve was included after each run to confirm only a single PCR product was generated for a given reaction. Samples were run in duplicate, and $2^{-\Delta\Delta Ct}$ was calculated for each sample, where $\Delta Ct$ = (Cttarget gene – Ctreference gene). Data were analysed as fold change in gene expression relative to the control group.

**Table 3 RT-PCR primers´ sequence details**

| Gene name | Forward primer | Reverse primer |
|---|---|---|
| TLR4 | ACATCAGAGGAAGAACAAGAAGCA | CGGAAATTGTAAACATAATGGGTTT |
| TNF-α | CTGGCCAATGGCATGGATCTCAAA | ATGAAATGGCAAATCGGCTGACGG |
| IL-1β | ACCTGCTAGTGTGTGATGTTCCCA | AGGTGGAGAGCTTTCAGCTCACAT |
| CXCL1 | AGAACATCCAGAGTTTGAAGGTGAT | GTGGCTATGACTTCGGTTTGG |
| HMGB1 | GAGGTGGAAGACCATGTCTG | AAGAAGAAGGCCGAAGGAGG |
| IL-6 | AAGCTGAGCGACGACTACAAGA | GTCAGCTCCAGCACCTTGTG |
| GAPDH | TGCACCACCAACTGCTTAGC | GGCATGGACTGTGGTCATGAG |

## 6.10 Behavioural Assessment

We focused on the evaluation of emotional and cognitive domains under the control of the frontal cortex (Rudebeck et al., 2008). The behavioural assessment was scheduled by sufficient time interval to avoid interferences with them, and they were performed during the dark phase (see Figure 2C for experimental timeline). Rats were evaluated following an alternation of experimental groups in all tests. The analysis was performed by a double-blind protocol to ensure the truthfulness of the results.

### 6.10.1 EMOTIONAL ASSESSMENT

#### 6.10.1.1 Anxiety-like behaviour

##### Elevated Plus Maze

To check anxiety-like behaviour, we performed the Elevated Plus Maze (EPM) test 12h after the ethanol binge. EPM is based on a balance of fear and curiosity towards novelty, and it is designed to test general anxiety-related behaviours in rodents (Cosquer et al., 2005; Pellow et al., 1985). The EPM was performed on two open black and grey plastic arms (50 x 10 cm) and two perpendicular enclosed arms of the same size but with opaque walls 50 cm high. The junction of the four arms formed a central square area (10 $cm^2$). The apparatus was elevated 65 cm above the floor. The light intensity was set up at 20 lux. On the test day, each rat was placed on the central platform facing one closed arm and opposite to the experimenter position. Then, the animal was allowed to freely explore the maze for 5 min. Between tests, the maze was carefully cleaned with ethanol 5% to remove possible odour from previous animals. Some animals fell from the EPM

and were excluded from the analysis. The number of entries and the time spent in all the arms were measured by a computer-controlled system (Mazesoft-4) recording the interruptions of infrared photo beams located along each arm. The percentage of each was calculated upon total entries into any arms and upon the total time spent in both arms, respectively. It was considered a visit whenever an animal entered an arm with all four limbs. Anxiety-like behaviour was defined as a decrease in the number of entries and time spent in the opened arms related to the total entries and total time, respectively, compared with control animals.

#### Open Field Test

The open-field test (OFT) is traditionally used to explore locomotor activity in rodents (Bouwknecht et al., 2007; Knight et al., 2021; Moya et al., 2021) but also widely used to study anxiety-like behaviour by checking specific behaviours in the field. The apparatus is a square black box (80 × 80 × 42 cm) with black matte-painted walls and floor and the base divided into 16×16 cm equal squares with legible white lines.

Each rat was placed in the corner of the floor and then allowed to freely explore the arena for 5 minutes. The behaviour of each rat was recorded on video and specific parameters were registered using an automatic monitoring system (Smart Software, 7.4 ANY-maze). The base was divided into two regions: the centre (inner zone) and the periphery (in contact with the walls), on the software screen. The frequency of the following behaviours was recorded: time spent in the inner zone (s), number of entries to the centre zone, number of grid crossings and rearing (standing on hind legs, with or without contact with the sides of the arena). This test was performed under white light conditions (20 lux). Anxiety-like behaviour was defined as decrease in the time spent in the inner zone and a decrease in the number of entries to the centre zone, grid crossings and/or rears, compared with control animals.

### 6.10.1.2 Depressive-like behaviour

#### Forced Swimming Test

The Forced Swimming Test (FST) is based on the method described by Porsolt (Porsolt RD et al., 1977) and it is one of the most common assays for the study of depressive-like behaviour in rodents (Slattery & Cryan, 2012; Yankelevitch-Yahav et al., 2015), but also see (Armario, 2021). 27h after the last ethanol administration (Figure 2C), animals were placed individually into transparent cylinders (47 x 35 cm) filled with water (25 ± 1ºC) for 5 min. Escape-directed behaviours were analysed, such as swimming, horizontal movements throughout the water tank; climbing, vertical movements of the forepaws; immobility and latency to immobility. All the

assays were performed under red light conditions and recorded for subsequent analysis. Either an increase in the immobility or a decrease in the swimming, climbing and latency times were considered depressive-like behaviour, reflecting a failure of persistence in escape (Cryan et al., 2002; Detke et al., 1995).

#### Saccharin Preference Test

The Saccharin Preference Test is used to measure anhedonia, which is a notable decline in the interest or pleasure derived from activities that were previously considered enjoyable. It is considered a core symptom of a depressive-like behaviour (Scheggi et al., 2018; Slattery et al., 2007). As animals were finishing the FST, they were dried with a towel and housed individually in cages provided with food *ad libitum.* For the Saccharin Preference test, rats were offered a free choice between 2 bottles located in the cage, one with 0.1% saccharin (w/v) and another with tap water. Bottles were weighed to determine the liquid consumption and placed into the cage with an alternated position of the water vs. saccharin to avoid place preference at specific intervals after the last ethanol administration (36h, 54h, 60h). The saccharin preference was calculated as the percentage of consumed saccharin over the total amount of liquid intake.

### 6.10.2 COGNITIVE ASSESSMENT

#### 6.10.2.1 Memory

#### Spatial memory: Morris Water Maze

The Morris Water Maze (MWM) test was used to measure spatial learning and memory for rodents, which is considered a type of declarative memory as animals are capable of acquiring the ability to navigate the designated route within a specific maze environment (Rendeiro et al., 2009).

It was carried out during 5 consecutive days in a circular pool (Figure 9) (diameter 122 cm). The water in the tank was made opaque with white non-toxic tempera paint (temperature 24 ± 1ºC). The pool was in a room with visible external cues and light intensity controlled. The experimenter worked also as a cue. A platform (diameter 10 cm) was submerged 1-2 cm below the water surface in one of four equal imaginary quadrants. During 4 consecutive days, animals were trained to find the submerged platform in a fixed location of the MWM. Each day consisted of 4 trials in which animals were released facing the wall from different points. Each trial had a maximum latency of 60 s, where animals freely explored the swimming pool to reach the platform. All rats were allowed to stay on the platform for 10 s more before being removed from

the water. Latencies to find the platform were recorded in each trial and the average was calculated for each day and animal. After each trial, rats were dried and returned to their home cages.

The fifth day, the test was carried out without the platform for 60 s with a new start position in the pool to ensure that the animals remember the goal location rather than a specific swim path. Here, we measured the latency to reach the previous platform location, the number of platform-site crossovers and the time spent within an imaginary ring (diameter 30 cm) around where the platform had been. All the assays were recorded by a video camera located above the pool for further analysis (Vorhees & Williams, 2006).

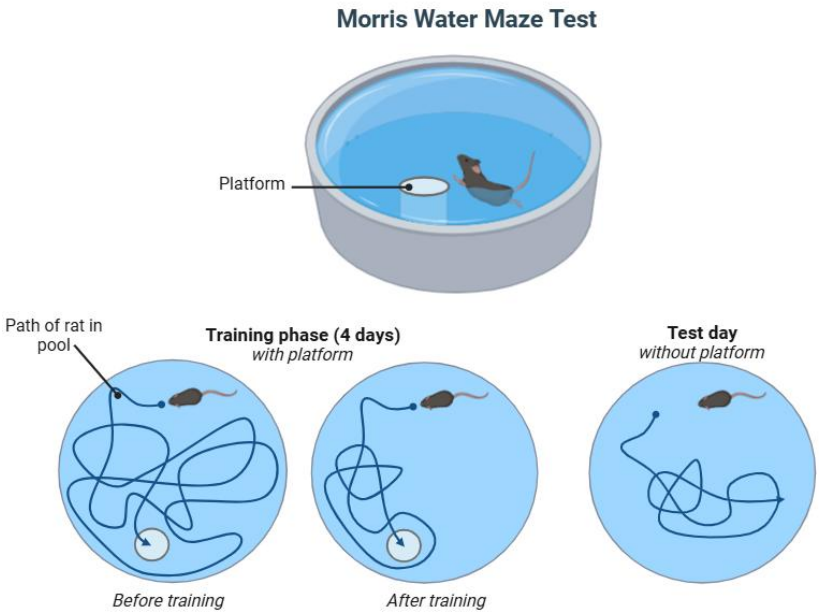



**Figure 9. Graphical illustration of Morris Water Maze performance over the five-day period (***Modified from BioRender).*

## Recognition memory: Novel Object Recognition Test

The Novel Object Recognition Test (NOR) was performed to study possible memory impairments based on the tendency of rodents to interact more with a novel object than a familiar one (Bevins & Besheer, 2006). Recognition memory is also categorised as a form of declarative memory and it refers to the cognitive ability to distinguish between familiar and unfamiliar items and to accurately identify them (Engelmann et al., 2011).

The test was performed in a square arena (80x80x42cm) with black matte-painted walls and floor. The arena was subdivided into 4 equal sections, allowing the evaluation of 4 rats simultaneously. The NOR was carried out in accordance with previous studies (Marco et al., 2013;

Moya et al., 2022) under low-light conditions (20 lux). The test (Figure 10) was organized in three phases: habituation (time = 0), a training phase (pre-test) and two test sessions 4h and 24h after the training phase. During the habituation, animals were allowed to freely explore the arena during 5 minutes without objects. In the training phase, 2 identical objects (glass bottles) were in opposite corners of the arena, and animals were allowed to freely explore them for 3 min. During the test session 4h after the training phase, one of the familiar objects (F, glass bottle) was substituted by a novel object (N1, green ashtray), and the rats were allowed to explore both objects for 5 min. In the 24h session, the novel N1 was replaced by another novel object (N2, money box) and the object positions in the arena were alternated in order to avoid possible place preferences. Both the training and test sessions were video recorded (Sony DCRDVD310E, Spain). Exploration of an object was considered whenever animals pointed their nose toward an object at a distance 1 cm, while turning around, climbing or biting the objects was not considered exploration. The latency to first explore the novel object in the test sessions was registered, and the discrimination index (DI) calculated as the difference between the time spent exploring the novel object and the familiar one in relation to the total time spent exploring the objects.

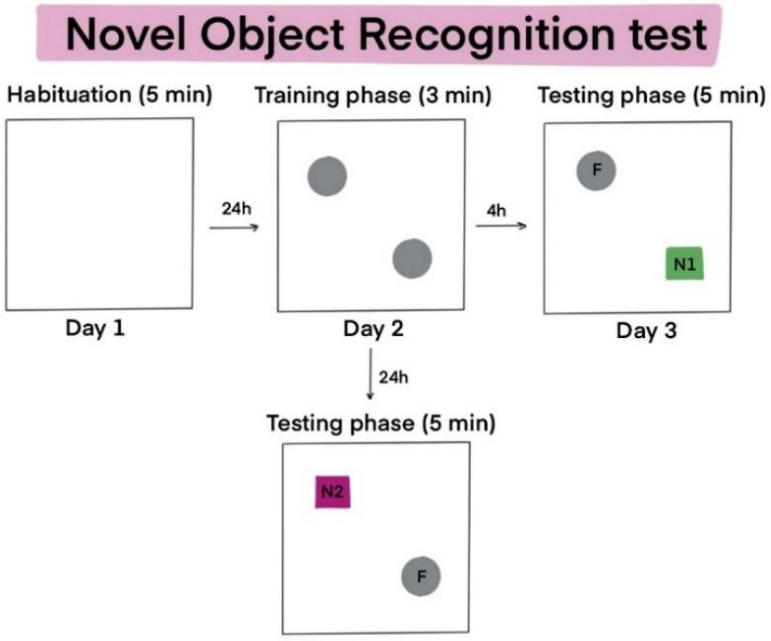
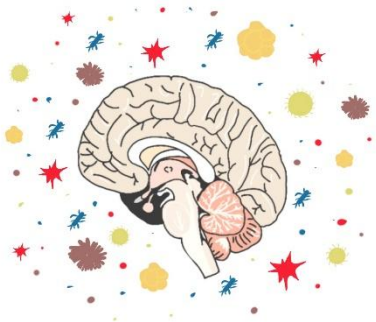



**Figure 10. Schematic diagram illustrating the recognition memory test assessed** *(own source, generated using Procreate). F*=familiar object; *N1*=novel object 1; *N2*=novel object 2.

## 6.11 Statistical analysis

All data are expressed as the mean ± S.E.M. Data from ELISA kits, qPCR and behavioural studies were analysed using a 2-way ANOVA to compare three or more experimental groups, comparing the factors [alcohol/water] versus sex [male/female] or [alcohol/water] versus treatment, respectively, when normality was verified; otherwise, a Kruskal-Wallis test was used. The saccharin preference test was analysed using a three-way ANOVA with time (36h, 54h, 60h), EtOH treatment (water versus alcohol) and HDL/4F pre-treatment (HDL or 4F versus vehicle) as independent factors. *Post hoc* comparisons (Bonferroni or Dunn's) were performed in case of significant interaction between factors, and only in instances where the Bonferroni *post hoc* did not resolve the interaction outcome has Tukey's method been employed. Homoscedasticity was checked by Barlett's test, and data were transformed (sqrt, log10) when appropriate. Data from western blots of each sex were analysed independently, comparing alcohol-treated animals versus controls by using the parametric Student's *t*-test or the nonparametric Mann–Whitney test, due to the samples from each sex were loaded in different blots. The outliers were analysed using Grubbs' test. Correlations were assessed by Pearson's and linear regression analyses. Animals that died during the experiments were excluded from all measurements and statistical analyses. A $p$ value $< 0.05$ was set as the threshold for statistical significance in all statistical analyses. All data were analysed using GraphPad Prism version 8.01 (GraphPad Software, Inc., La Jolla, CA).

# 7. RESULTS



# 7.1 STUDY I: STUDY OF THE PRESENCE OF BACTERIAL COMPONENTS IN THE BRAIN OF MALE AND FEMALE ANIMALS SUBJECTED TO INTENSIVE ALCOHOL CONSUMPTION

This study has been published in Journal of Lipid Research (López-Valencia et al., 2024) and is attached as an annex at the end of this manuscript.

## 7.1.1 Effects of the binge drinking model in male and female rats

### 7.1.1.1 Plasma corticosterone and BELs in male and female rats

This IAC protocol has been widely used in our lab (Antón et al., 2017; Rodríguez-González et al., 2021) and by other authors (Obernier, Bouldin, et al., 2002; Obernier, White, et al., 2002) to model ABD in males. In this Doctoral Thesis, we used the model in male rats and, for the first time, in female rats. Thus, in this section, the characteristics of this animal model are described, as well as some of the sexual differences found.

The number of rats per group was initially higher in the alcohol groups, since this protocol often induces mortality. In this experiment, the mortality was around 7%, similar to other studies (Antón et al., 2017; Obernier, Bouldin, et al., 2002; Rodríguez-González et al., 2021).

We checked BELs and plasma corticosterone in males and females 3h after the last binge administration (Table 4). The mean BELs found in males were within the binge drinking definition, in accordance with previous literature mentioned above, whereas females reached lower BELs (< 80 mg/dL; Table 4, Student´s *t*-test, $p<0.05$).

Regarding corticosterone levels, an interaction between factors was found ($F_{(1, 30)} = 10.01$, $p=0.0036$) with overall ethanol and sex effects ($F_{(1, 30)} = 6.165$; $p=0.0188$; $F_{(1, 30)} = 7.282$; $p=0.0113$, respectively). *Post hoc* comparisons revealed that corticosterone was not altered during experimental conditions in males, but it was increased in female alcohol-treated animals versus female controls ($p<0.01$).

**Table 4. Plasma corticosterone and BELs in male and female rats**

| | Males | | Females | | | |
|---|---|---|---|---|---|---|
| | **Control** | **EtOH** | **Control** | **EtOH** | **Student´s *t*-test** | 2-way ANOVA |
| **BELs (mg/dL)** | --- | 101.52 ± 19.61 | --- | 56.95 ± 17.09* | $p$=0.0491 | --- |
| **Corticosterone (ng/mL)** | 186.53 ± 38.74 | 156.11 ± 15.81 | 165.74 ± 22.97 | 418.05 ± 71.83*& | **----** | Overall alcohol effect: $^{\#}p$<0.05 Overall sex effect: $^{\$}p$<0.05 Interaction (*post hoc* test): *$^{\&}p$<0.05 |

There was an interaction between alcohol and sex in the levels of corticosterone: alcohol-treated females had higher corticosterone levels than the female controls (Bonferroni *post hoc* test: *$p$<0.05) and alcohol-treated males (Bonferroni *post hoc* test: $^{\&}p$<0.05). BELs (blood alcohol levels achieved 3h after the last ethanol gavage) differed between male and female animals (Student´s *t*-test, *$p$<0.05). Data are shown as the mean ± S.E.M. Statistical analysis: 2-way ANOVA: overall effect of ethanol: $^{\#}p$<0.05; overall effect of sex: $^{\$}p$<0.05; interaction between factors (alcohol/sex), followed by Bonferroni *post hoc* test: *$p$<0.05. *EtOH*=ethanol-treated animals.

### 7.1.1.2 Hepatic enzymes involved in alcohol metabolism

We explored how ethanol binges affected to the expression of the hepatic enzymes involved in the alcohol metabolism: ADH and ALDH, since some basal differences have been already described in male and female rats (Elena Quintanilla et al., 2007).

Results are expressed as percentages of control male animals (ADH: 26.71 ± 1.77 ng/mg protein; ALDH: 1.25 ± 0.02 ng/mg protein). ADH showed a tendency to increase in the ethanol-treated groups versus control groups, although it did not reach statistical significance (Fig. 11A; $F_{(1, 30)} = 3.751$; $p$=0.0622). No sex main effect or interaction between factors (*alcohol x sex)* were found ($F_{(1, 30)} = 2.543$; $p$=0.1213; $F_{(1, 30)} = 3.007$; $p$=0.0932, respectively).

Regarding ALDH, we did not observed an alcohol or sex main effect (Fig.11B; $F_{(1, 29)}$ = 0.7140; $p$=0.4050; $F_{(1, 29)} = 1.285$; $p$=0.2662, respectively). However, an interaction between factors was found ($F_{(1, 29)} = 10.89$; $p$=0.0026). Bonferroni *post hoc* test revealed differences between female control group vs. male control group ($p < 0.05$) and vs. ethanol-treated females ($p < 0.05$).

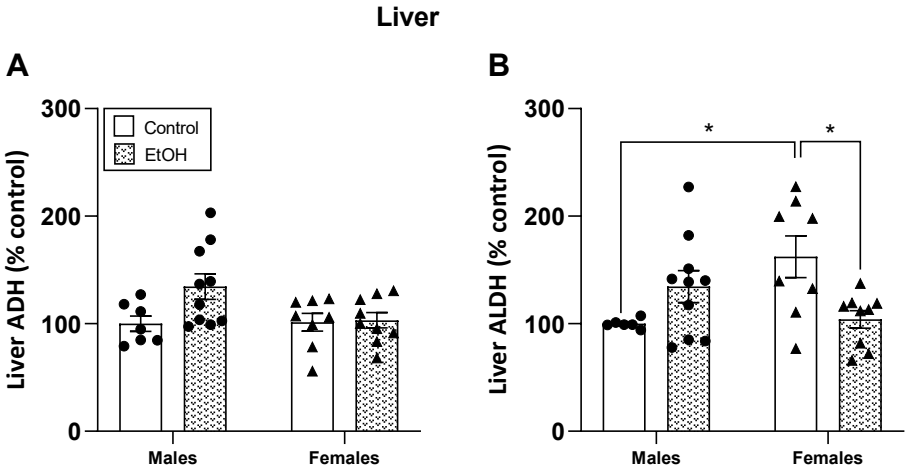



**Figure 11. Hepatic enzymes involved in alcohol metabolism in rats which underwent alcohol binges.** **A** Expression of liver ADH in control and ethanol-treated male and female rats. **B** Expression of liver ALDH in control and ethanol-treated male and female rats. All data are expressed as mean ± S.E.M. Two-way ANOVA followed by Bonferroni *post hoc* test: $^{*}p < 0.05$. Results are expressed as percentage of control male animals. *ADH*=alcohol dehydrogenase; *ALDH*=aldehyde dehydrogenase; *EtOH*=ethanol-treated animals.

### 7.1.1.3 Determination of the estrous cycle phases in female rats

In females, the determination of the estrous cycle of each rat was done along the 4 days of experimentation. The distribution of phases in each animal was aleatory and no synchronization was observed among them (Table 5). Figure 12 shows a schematic representation of the distribution of the phases of the estrous cycle.

**Table 5. Distribution of estrous cycle phases in female rats along ABD procedure**

| Experimental group | Day 1 | Day 2 | Day 3 | Day 4 |
|---|---|---|---|---|
| CONTROL (n=8) | DIESTRUS | PROESTRUS | ESTROUS | METESTRUS |
| | ESTROUS | METESTRUS | DIESTRUS | DIESTRUS |
| | DIESTRUS | DIESTRUS | PROESTRUS | ESTROUS |
| | DIESTRUS | DIESTRUS | PROESTRUS | ESTROUS |
| | ESTROUS | METESTRUS | DIESTRUS | PROESTRUS |
| | DIESTRUS | ESTROUS | ESTROUS | METESTRUS |
| | METESTRUS | METESTRUS | DIESTRUS | PROESTRUS |
| | METESTRUS | DIESTRUS | PROESTRUS | ESTROUS |
| ETOH (n=9) | METESTRUS | METESTRUS | DIESTRUS | DIESTRUS |
| | PROESTRUS | ESTROUS | DIESTRUS | DIESTRUS |
| | METESTRUS | METESTRUS | DIESTRUS | PROESTRUS |
| | ESTROUS | ESTROUS | METESTRUS | METESTRUS |
| | DIESTRUS | ESTROUS | METESTRUS | METESTRUS |
| | METESTRUS | DIESTRUS | PROESTRUS | ESTROUS |
| | DIESTRUS | PROESTRUS | ESTROUS | METESTRUS |
| | ESTROUS | METESTRUS | METESTRUS | DIESTRUS |
| | DIESTRUS | PROESTRUS | ESTROUS | METESTRUS |

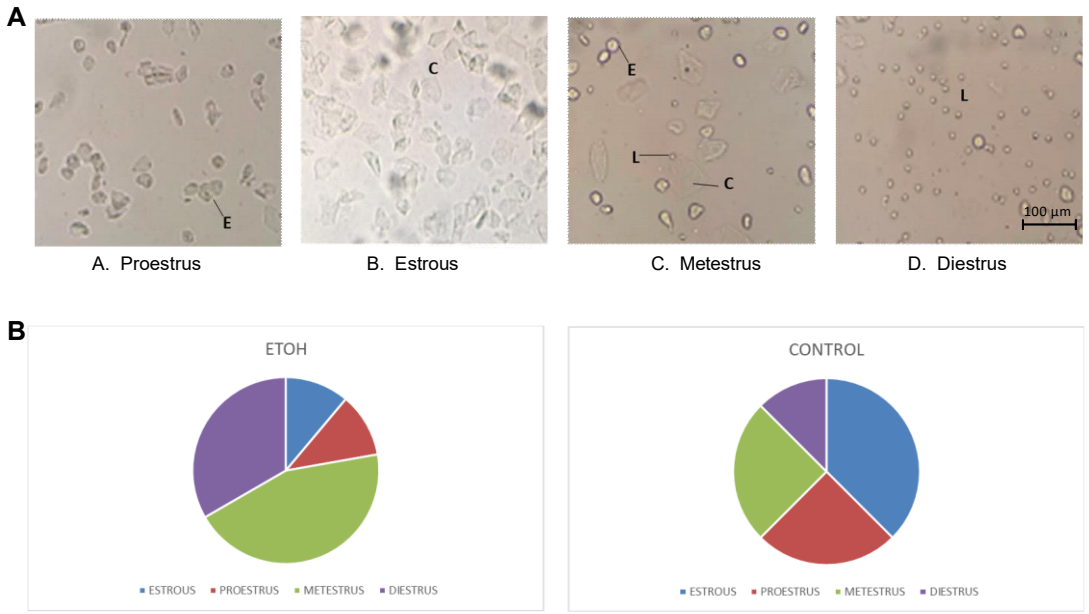



**Figure 12. Determination of the estrous cycle. A. Schematic representation of unstained vaginal smear from females' cycle phases**. The proportion of three types of cells was used as a determinant of the cycle phases: epithelial cells (E), nucleated and round; cornified cells (C), without nucleus and irregular form; and leukocytes (L), round tiny ones. **A** Proestrus, with a predominance of epithelial cells. **B** Estrous, consisting mainly of cornified cells. **C** Metestrus, consists of the same proportion of the three types of cells. **D** Diestrus, with a predominance of leukocytes. **B. Circle diagram shows the percentage distribution of estrous cycle in the ethanol and control female animals at the end of the experiment (day 4).** *EtOH*=ethanol-treated animals.

### 7.1.2 Bacterial products in the plasma of alcohol-intoxicated males and females and control animals: plasma LPS and LBP

LPS was detectable in plasma in control animals both in male (0.491±0.087 EU/mL) and female rats (0.612±0.079 EU/mL). Although it is known that ABD induces an increase in the plasma LPS levels in males with the same protocol (Antón et al., 2017), in our analyses including both sexes we did not find significant alterations in LPS levels (no interaction between factors: $F_{(1, 29)} = 0.01027$, $p = 0.9200$). However, it is to note that an overall ethanol effect near of significance was found in both ethanol-treated groups (Fig. 13A; $F_{(1, 29)} = 3.441$, $p = 0.0738$), with no sexual differences ($F_{(1, 29)} = 0.4960$; $p = 0.4869$, respectively).

Nonetheless, we observed significant elevations in plasma LBP, a protein related with the signalling of bacterial LPS, in response to ethanol binges, both in males and females. LBP has also been considered a marker of bacterial translocation and inflammation (Stehle et al., 2012) and it was elevated in the ethanol-treated groups versus control groups (Fig. 13B; 2-way ANOVA, overall alcohol effect: $F_{(1, 28)} = 6.436$, $p$=0.0170). No sexual differences were found.

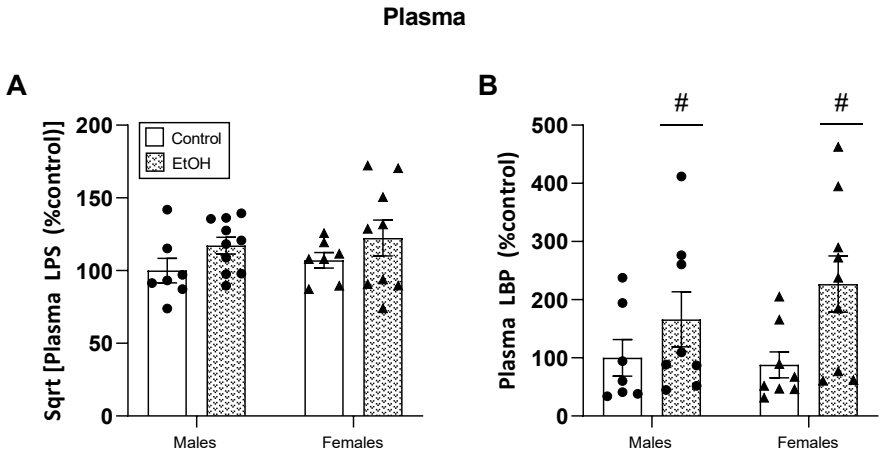



**Figure 13. Expression of LPS and LBP in the plasma of male and female ethanol-treated and control animals**. **A** LPS levels. **B** LBP levels. Statistical analysis: 2-way ANOVA: overall effect of ethanol: $^{\#}p <$ 0.05. *LPS*=lipopolysaccharide; *LBP*=LPS-binding protein; *EtOH*=ethanol-treated animals.

### 7.1.3 Effects of the binge drinking model in the expression of plasma apolipoproteins and lipoproteins

#### 7.1.3.1 Expression of Apolipoproteins (AI, B and E) in plasma of alcohol-intoxicated males and females and control animals

Regarding ApoAI levels in plasma (Fig. 14A), control males showed similar basal levels (529.15 ± 41.53 µg/mL) than control females (498.44 ± 62.68 µg/mL). 2-way ANOVA reported a significant interaction between ethanol and sex (Fig. 14A; $F_{(1, 31)} = 4.376$, $p$=0.0447) with an overall ethanol effect ($F_{(1, 31)} = 4.489$; $p$=0.0422) and a sex effect near of significance ($F_{(1, 31)} =$ 3.368, $p$=0.0761). *Post hoc* comparisons revealed that plasma ApoAI was elevated in female alcohol-treated rats versus female controls and versus alcohol-treated males (Fig. 14A, $p$<0.05 in both cases).

Plasma ApoB (Fig. 14B) showed an overall sex effect ($F_{(1, 30)} = 16.40$, $p$=0.0003) and an interaction between factors ($F_{(1, 30)} = 4.762$, $p$=0.0371) after 2-way ANOVA. *Post hoc* test revealed a basal sexual dimorphism with control females showing lower levels of plasma ApoB (684.19 ± 1.18 µg/mL) than control males (1206.68 ± 107.29 µg/mL) ($p$<0.05) but no specific effect of alcohol ($F_{(1, 30)} = 0.4913$, $p$=0.4888, n.s.).

ApoE levels in plasma were under detection limits in the enzyme-link immunosorbent assay in all experimental and control groups tested.

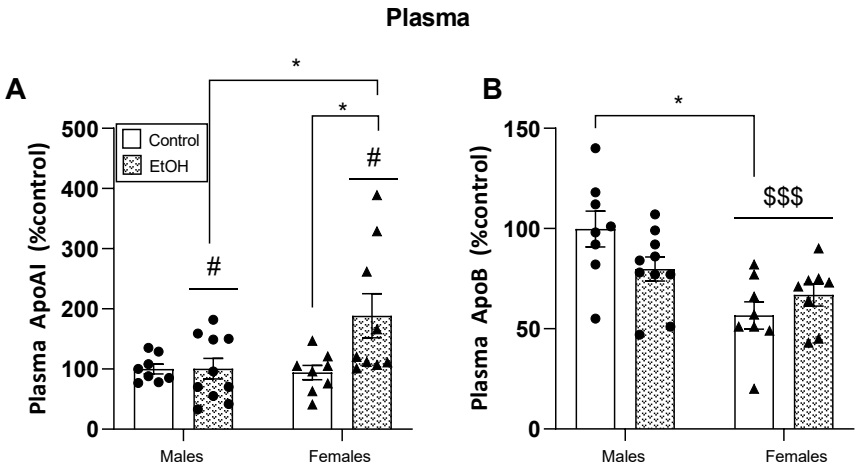



**Figure 14. Expression of apolipoproteins in the plasma of male and female ethanol-treated and control animals**. **A** Plasma ApoAI. **B** Plasma ApoB. No detectable levels for plasma ApoE. All data are expressed as mean ± S.E.M. Statistical analysis: 2-way ANOVA: overall effect of ethanol: $^{\#}p < 0.05$; overall effect of sex: $^{\$\$\$}p < 0.001$; interaction between factors (alcohol/sex), followed by Bonferroni *post hoc* test: $^{*}p < 0.05$. *EtOH*=ethanol-treated animals.

### 7.1.3.2 Expression of lipoproteins (HDL, LDL) in plasma of alcohol-intoxicated males and females and control animals

Since each Apo studied in this section is mainly incorporated into lipoproteins of different densities, we quantified the plasma levels of HDL (which incorporates mainly ApoAI and ApoE) and LDL (which incorporates mainly ApoB).

The results of plasma HDL and LDL are shown in Table 6. A 2-way ANOVA found no interaction between factors for HDL ($F_{(1, 31)} = 0.6047$, $p$=0.4427) and no overall alcohol ($F_{(1, 31)} = 0.7839$, $p$=0.3828) or sex ($F_{(1, 31)} = 0.7870$, $p$=0.3819) effects. Regarding LDL, the 2-way ANOVA indicated an overall effect of sex ($F_{(1, 31)} = 9.449$, $p$=0.0044) and no alcohol effect ($F_{(1, 31)} = 2.626$, $p$=0.1152) or interaction ($F_{(1, 31)} = 0.9269$, $p$=0.3431), revealing a basal sexual dimorphism with males showing elevated plasma LDL than females (Table 6).

**Table 6. Plasma HDL and LDL in control and alcohol-treated animals**

| | Males | | Females | | | |
|---|---|---|---|---|---|---|
| | **Control** | **EtOH** | **Control** | **EtOH** | **Student´s *t*-test** | 2-way ANOVA |
| **HDL (µg/mL)** | 517.23 ± 43.30 | 415.08 ± 63.69 | 414.98 ± 60.73 | 408.36 ± 68.51 | --- | n.s. |
| **LDL (µg/mL)** | 171.10 ± 6.27 | 138.20 ± 15.24 | 119.70 ± 12.18 | 111.32 ± 13.02 | --- | Overall sex effect: $^{\$\$}p<0.01$ |

No differences between males and females were detected in plasma HDL. An overall effect of sex was found in LDL levels, with higher levels in males. Data are shown as the mean ± S.E.M. Statistical analysis: 2-way ANOVA: overall effect of sex: $^{\$\$}p<0.01$. *EtOH*=ethanol-treated animals.

### 7.1.4 Presence of LPS components (Lipid A and Core) in the PFC of alcohol-intoxicated males and females and control animals

It is well-known that LPS is a component of the outer membrane of Gram-negative bacteria which may translocate to blood from the gut under certain conditions (Erridge et al., 2002; Vargas-Caraveo et al., 2017). It is a big molecule and so its possible permeability to the brain is controversial due to its size. For this reason, the smaller components of LPS, Lipid A and Core, were measured in this study in the brain (PFC and cerebellum) of male and female animals treated with alcohol binges and their control groups. In this section, we show the specific expression within the PFC (results of cerebellum showed in section 7.1.8).

Interestingly, both Lipid A and Core, in their free (non-bound) forms, were detectable and measurable within the PFC of males and females in both experimental groups. Western blots were run independently for male and female animals, so the variable sex was not included in the statistical analysis of this experiment. Data is presented as percentage of change over each respective control group and analysed accordingly.

Figure 15 shows the expression of LPS components in the PFC of male (upper panel, Fig.15A B, C) and female (lower panel, Fig. 15D, E, F) animals. There were no significant differences in the expression of Lipid A or Core levels (Fig. 15A,B; $t_{(16)}=0.3174$, $p=0.7551$; $t_{(16)}=0.4200$, $p=0.6801$, respectively) between the ethanol and control groups in males. Representative blots are shown in Fig. 15C.

In females, there were no changes in Lipid A and Core between the control and alcohol-treated groups (Fig. 15D,E; Mann–Whitney U=21; $p>0.05$, n.s.; $t_{(15)}=0.7107$, $p=0.4881$, respectively). Representative blots are shown in Fig. 15F.

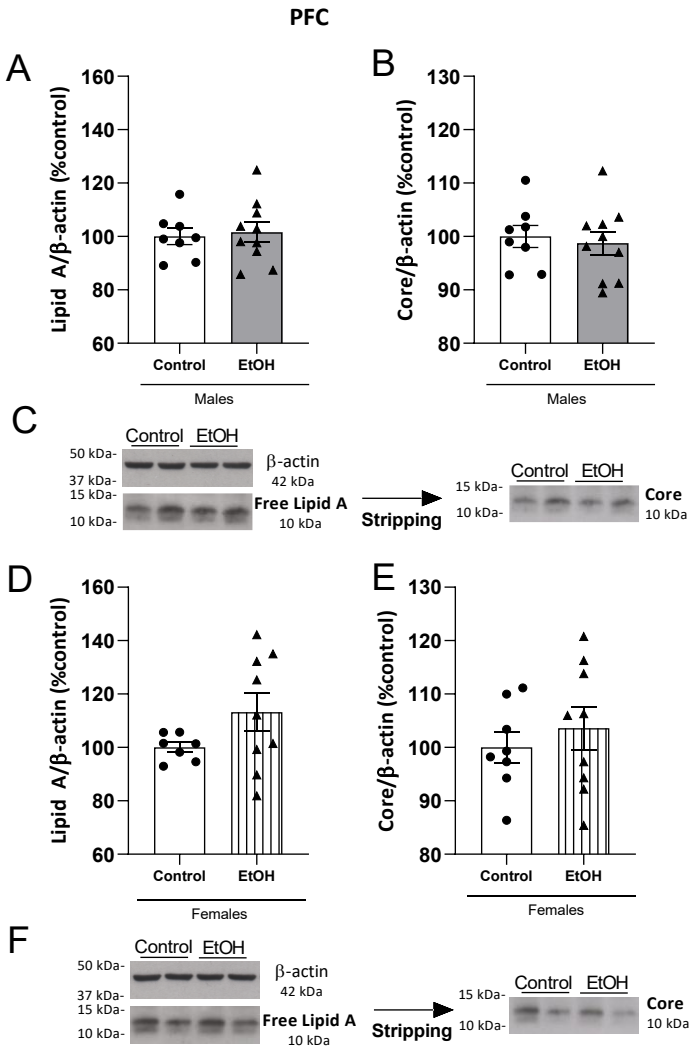



**Figure 15. Detection of the LPS components (Lipid A and Core) in prefrontal cortex by western blotting.** The upper panel shows data in ethanol-treated (n=10) and control (n=8) males and the lower panel data in ethanol-treated (n=9) and control (n=8) females. **A** Expression of Lipid A in males in PFC. **B** Expression of the core element of LPS in males. **C** Representative immunoblots of Lipid A and core from the same gel in males. **D** Expression of Lipid A in females in PFC. **E** Expression of the core element of LPS in females. **F** Representative immunoblots of Lipid A and core from the same gel in females. Western blot data were normalized by β-actin and expressed as a percentage of change over controls. Results represent the mean ± S.E.M. of two technical replicates. No differences were observed between groups. Student's *t*-test. *EtOH*=ethanol-treated animals.

#### 7.1.4.1 Screening study of the binding of Lipid A to different molecules in the PFC: pilot study with TLR4

After studying the expression of Lipid A and Core in the PFC in their free forms, our goal was to investigate the possible binding of these elements to different apolipoproteins within the brain. We chose Lipid A (the LPS domain considered endotoxic and proinflammatory) for this colocalization study.

In a pilot study, we used samples of male control and ethanol-treated animals to characterize the pattern of LPS binding to different molecules. We detected that Lipid A showed expression by western blot at different molecular weights, which corresponded with receptors and apolipoproteins to which it may bind, as suggested in previous publications (Vargas-Caraveo et al., 2017). Figure 16A shows the complete profile of Lipid A expression (free and bound to other proteins) by western blot when incubated with the antibody against Lipid A.

A positive control for Lipid A (15 µg of E. Coli LPS (O111:B4, ref. L2630 from Sigma-Aldrich, Spain) was used to detect free (not bound form) Lipid A and the rest of the bands are samples of male control and ethanol-treated animals. The free Lipid A (not bound form) which was visualized in a band at approximately 10 kDa (Fig. 16A). The antibody against Lipid A also showed other immunoreactivities at different molecular weights, indicative of the well-known binding of LPS to other molecules, as suggested before (Vargas-Caraveo et al., 2017). Specifically, we detected identifiable bands at ~31 kDa, ~48 kDa, ~75 kDa, ~96 kDa and ~210 kDa, which may correspond to the binding of Lipid A to ApoAI, CD14, SR-B1, TLR4 and ApoB, respectively (Fig. 16). Immunoblots of each of those proteins incubated with each specific antibody in each case are shown at the right of the panel in Fig. 16A.

To determine whether Lipid A was bound to some of these components, we used two approaches: 1) using western blot, we checked the expression of each mentioned protein at the specific molecular weight by incubating first with the antibody against Lipid A, which showed a specific band of certain kDa, and then we stripped the membrane and incubated with the antibody against the protein of interest (i.e. SR-BI), observing the same ubication of the band (same kDa); and repeated the action in an opposite order (first, the incubation with the specific antibody, and then the incubation with Lipid A antibody); 2) we confirmed the binding of the two proteins of interest by using co-IP technique.

Thus, as an example, Fig 16B and C represent the binding of Lipid A to TLR4. Fig. 16B shows the expression of TLR4 (band at ~96 kDa). The membrane was stripped and then incubated with the antibody against Lipid A. The results of the [LipidA-TLR4]-bound form were normalized by the total expression of TLR4 in the PFC (Fig. 16B).

The binding of Lipid A to TLR4 was confirmed twice by co-IP, by alternation the order of incubation of antibodies of interest (see method section 6.6 and 6.7). First, we immunoprecipitated with the antibody against Lipid A, and then the complex was incubated with the antibody against TLR4 (Fig. 16C, upper panel). To double check this binding, another set of

samples was prepared, and the conjugate was precipitated using an antibody against TLR4 first and visualized by analysis of the immunoreactivity against Lipid A (Fig. 16C, lower panel).

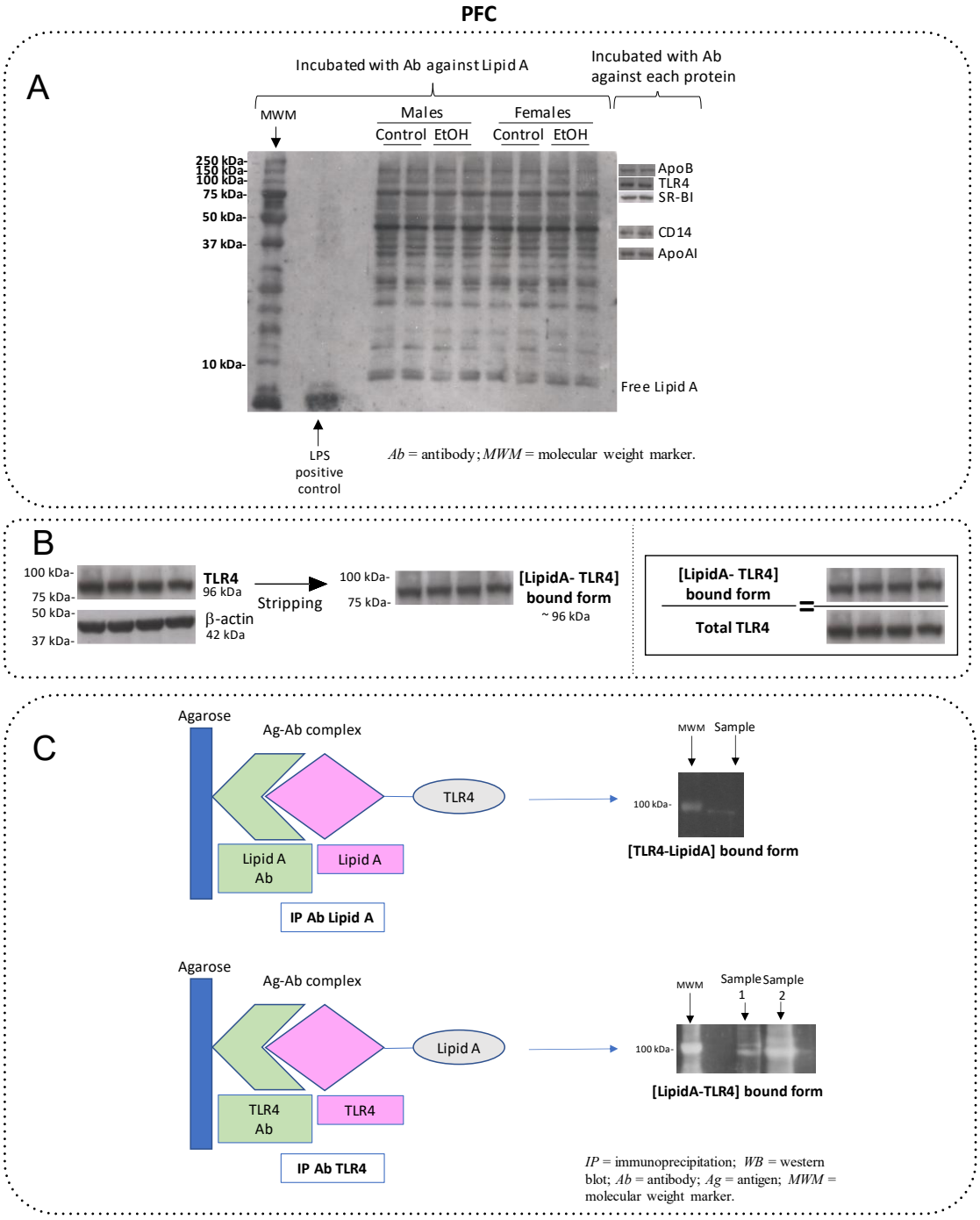



**Figure 16. Screening study of Lipid A bound to different molecules in PFC: pilot study with TLR4.** **A** Representative immunoblot of a membrane from a 18% gel incubated with antibody against Lipid A, where Lipid A showed expression at different molecular weights (left side). On the right side, samples of immunoblots which correspond with receptors and apolipoproteins which Lipid A may bind to. **B** Representation of the binding of Lipid A to TLR4. The ratio [LipidA-TLR4] bound form is an indirect measurement of co-localization of proteins. [LipidA-TLR4] bound form was detected at 96 kDa and

normalized by total TLR4. **C** Left panel in C is a schematic representation of the co-IP process as a direct measure of co-localization of Lipid A and TLR4. Upper panel represents the immunoreactivity obtained from Lipid A immunoprecipitated incubated with an antibody against TLR4. Down panel shows the immunoreactivity obtained from TLR4 immunoprecipitated incubated with an antibody against Lipid A. *EtOH*=ethanol-treated animals.

In this pilot study, we showed that our approach of binding of Lipid A and TLR4 by western blotting was confirmed by co-IP. In a new study, we used both western blotting and co-IP to check the binding of Lipid A to TLR4 and different apolipoproteins and their receptors, and the results are presented in the next sections. Co-IP was used as qualitative confirmation of each specific binding of proteins (conjugate first precipitated by Lipid A antibody and then the complex incubated with the antibody of the protein of interest), and we used western blot analyses to quantify the samples in our study, since the co-IP is limiting tissue technique that needs pooled samples (see method section).

In the next sections, we report the studies of the binding of Lipid A with TLR4, ApoAI, ApoB and ApoE in the different experimental groups.

### 7.1.5 Expression of apolipoproteins and its receptors and formation of aggregates with bacterial products in the PFC of animals exposed to alcohol binges

#### 7.1.5.1 Expression of free and bound forms of Apolipoprotein AI in the PFC and the SR-BI receptor in male and female animals

ApoAI was detectable in the PFC of control and ethanol-treated animals by western blotting (Figure 17). In male rats, there were no differences between the alcohol and control groups in total ApoAI levels in the PFC (Fig. 17A; $t_{(16)}$=0.2420, $p$=0.8119). Regarding the [LipidA-ApoAI] bound form, we did not observe significant differences between groups (Fig. 17B; $t_{(16)}$=1.209, $p$=0.2442). The data showed that SR-BI was detectable in this structure by western blot, with no changes between the ethanol and control groups (Fig. 17C; $t_{(16)}$=0.02170, $p$=0.9830). Fig. 17D shows the representative blots for these proteins in males.

In female rats, whereas no differences in total ApoAI levels were detected in the PFC (Fig. 17E; $t_{(15)}$=0.4364, $p$=0.6687), we observed an increase in the [Lipid A-ApoAI] bound form in the ethanol group compared to controls (Fig. 17F; Mann–Whitney, U=0, $p$<0.001). Expression of the ApoAI receptor SR-BI was not altered in females (Fig. 17G; $t_{(15)}$=0.6976, $p$=0.4961). Blots are represented in Fig. 17H for female animals.

Figure 17I shows a representative diagram of the precipitated complex in the co-IP procedure using pooled samples (left panel) and a representative image of the confirmation of the binding between Lipid A and ApoAI by co-IP in the PFC (right panel).

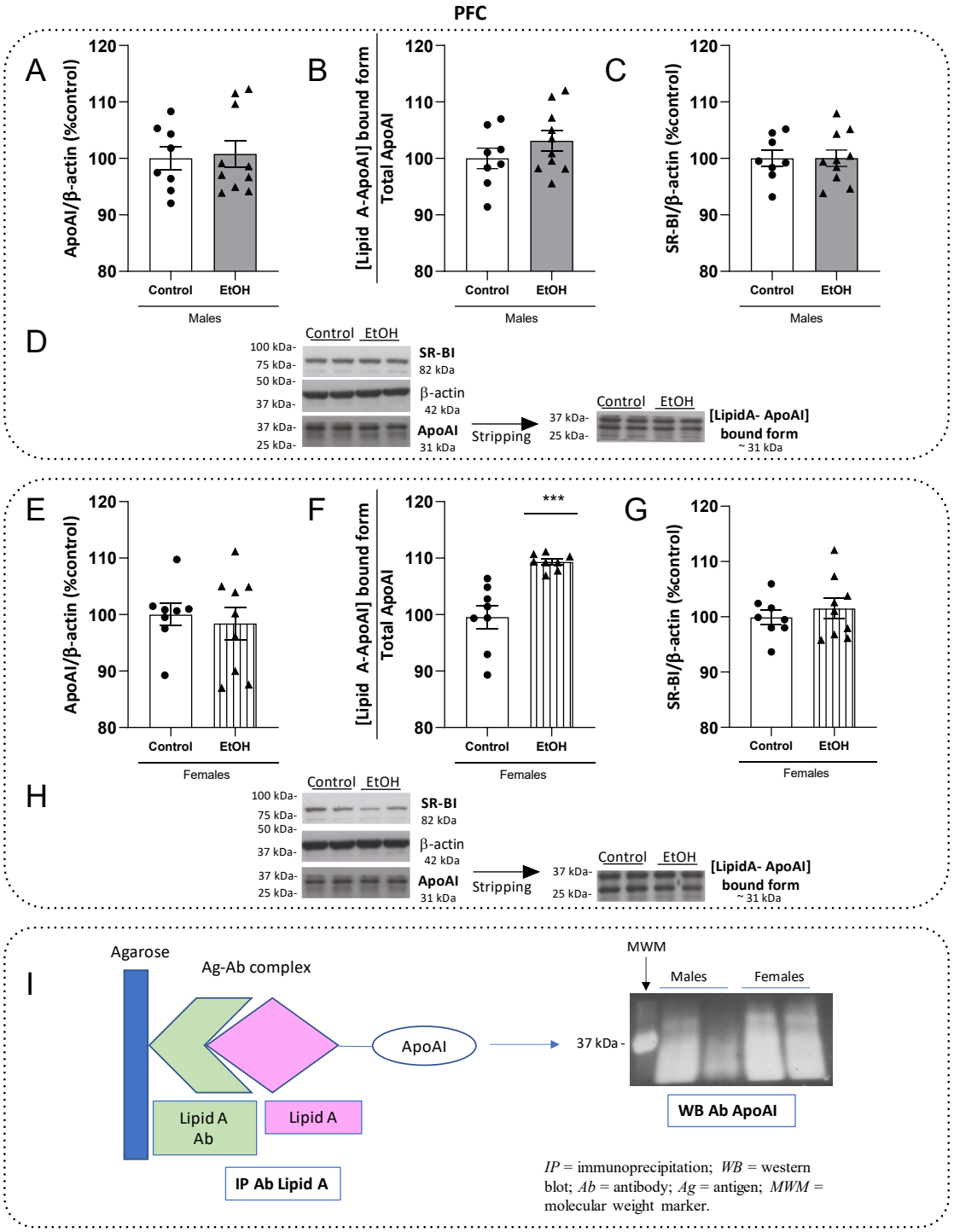



**Figure 17. Expression of ApoAI and [LipidA-ApoAI] by western blotting and its receptor SR-BI in PFC.** The upper panel shows data in ethanol-treated (n=10) and control (n=8) males and the lower panel data in ethanol-treated (n=9) and control (n=8) females. **A** Expression of total ApoAI in males. **B** The ratio [LipidA-ApoAI] bound form in males, as indirect measurement of co-localization of proteins (Lipid A detected at 31 kDa and normalized by total ApoAI). **C** Expression of SR-BI in males. **D** Representative

immunoblots of total ApoAI, [LipidA-ApoAI] bound form and SR-BI in males from the same gel. **E** Expression of total ApoAI levels in females. **F** The ratio [LipidA-ApoAI] bound form in female, as indirect measurement of co-localization of proteins, was increased in ethanol-treated females. Lipid A was detected at 31 kDa and normalized by total ApoAI. **G** Expression of SR-BI levels in female rats. **H** Representative immunoblots of total ApoAI, [LipidA-ApoAI] bound form and SR-BI in females from the same gel. **I** Co-localization of Lipid A and ApoAI by co-immunoprecipitation (co-IP). Letf panel in I is a schematic representation of the co-IP process. Co-IP is a direct measure of co-localization of Lipid A and ApoAI. Right panel shows a representative image of the co-IP. Results were obtained by pool of samples in the same experimental group and are descriptive. Quantification of data was done by western blotting, and it is shown in B and F. Results represent the mean ± S.E.M. of two technical replicates. Differences from control group: ***$p<0.001$ (Student's *t*-test or Mann-Whitney (3F)). *EtOH*=ethanol-treated animals.

#### 7.1.5.2 Expression of the free and bound forms of Apolipoprotein B in the PFC and the LDL receptor in male and female animals

ApoB levels were detectable in the PFC in all experimental groups, and quantifiable analyses are shown in Fig. 18. There were no significant differences in total ApoB levels between the alcohol and control groups in males (Fig. 18A; $t_{(15)}=1.381$, $p=0.1876$). However, we observed a significant increase in the [LipidA-ApoB] bound form in ethanol-treated male animals versus controls (Fig. 18B; $t_{(16)}=2.448$, $p=0.0263$), and interestingly, levels of the ApoB receptor LDLr in the PFC were also upregulated in the ethanol group in males (Fig. 18C; $t_{(16)}=2.253$, $p=0.0387$). Fig. 18D shows representative blots for these proteins in male rats.

In females, there were no significant changes between groups in total ApoB levels (Fig. 18E; $t_{(15)}=0.09525$, $p=0.9254$), the [LipidA-ApoB] bound form (Fig. 18F; $t_{(15)}=0.9816$, $p=0.3419$) or the expression of LDLr (Fig. 18G; $t_{(15)}=1.235$, $p=0.2360$). Fig. 18H shows the representative blots for these proteins in females.

The binding of Lipid A with ApoB was confirmed by visualization of the immunoprecipitated complex by co-IP in pooled samples (Fig. 18I).

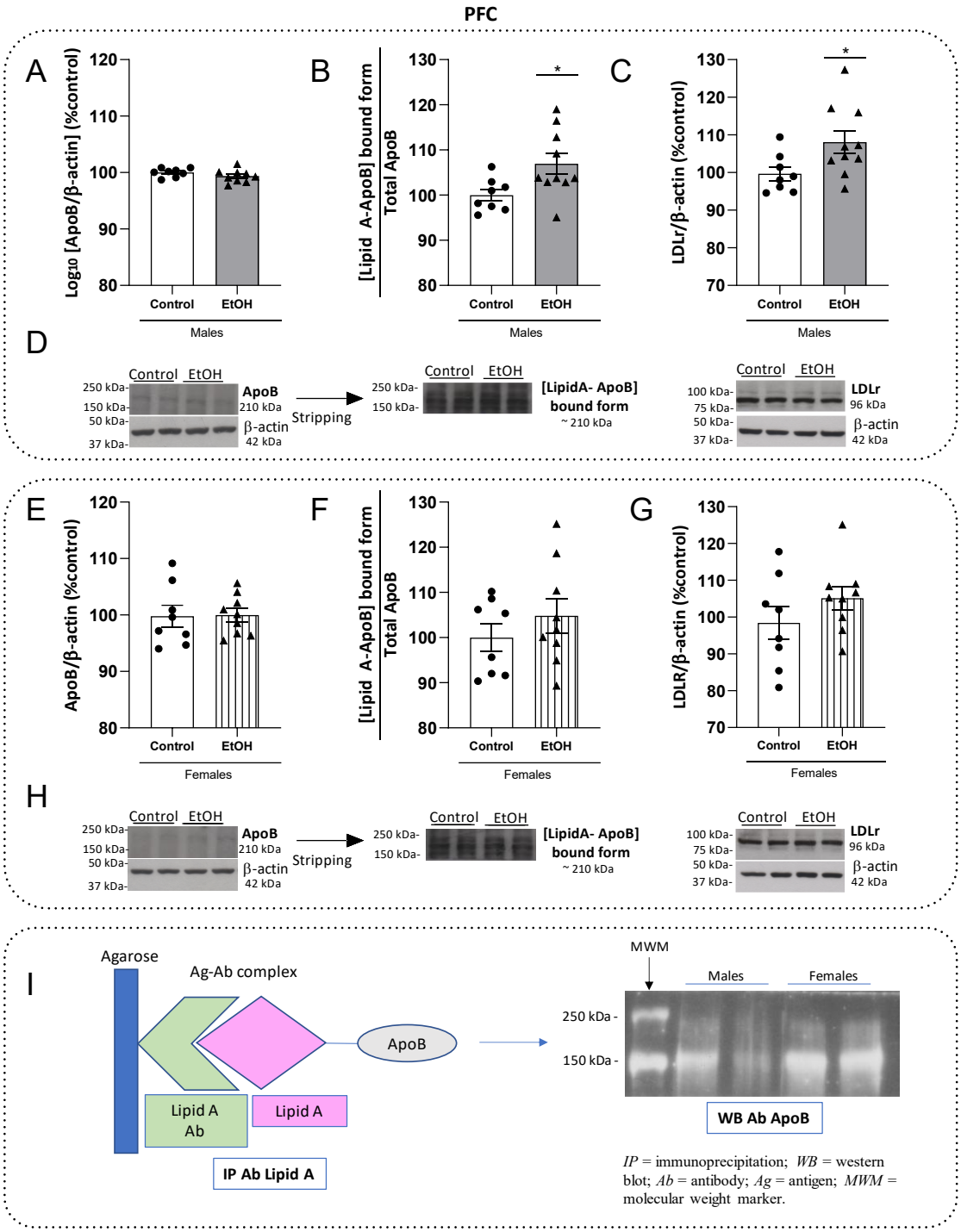



**Figure 18. Expression of ApoB and [LipidA-ApoB] by western blotting and its receptor LDLr in PFC.** The upper panel shows data in ethanol-treated (n=10) and control (n=8) males and the lower panel data in ethanol-treated (n=9) and control (n=8) females. **A** Expression of total ApoB in males. **B** The ratio [LipidA-ApoB] bound form in males, as indirect measurement of co-localization of proteins, was increased in ethanol-treated group. Lipid A was detected at 210 kDa and normalized by total ApoB. **C** LDLr levels were increased in ethanol group in males. **D** Representative immunoblots of total ApoB, [LipidA-ApoB] bound form and LDLr in males from different gels. **E** Expression of total ApoB levels in females. **F** The ratio [LipidA-ApoB] bound form in female, as indirect measurement of co-localization of proteins (Lipid A was detected at 210 kDa and normalized by total ApoB). **G** Expression of LDLr levels in female rats. **H** Representative immunoblots of total ApoB, [LipidA-ApoB] bound form and LDLr in females from

different gels. **I** Co-localization of Lipid A and ApoB by co-IP. Letf panel in I is a schematic representation of the co-IP process. Co-IP is a direct measure of co-localization of Lipid A and ApoB. Right panel shows a representative image of the co-IP. Results were obtained by pool of samples in the same experimental group and are descriptive. Quantification of data was done by western blotting, and it is shown in B and F. Results represent the mean ± S.E.M. of two technical replicates. Differences from control group: *$p<0.05$ (Student's *t*-test). *EtOH*=ethanol-treated animals.

#### 7.1.5.3 Expression of the free and bound forms of Apolipoprotein E in the PFC and the ApoER2 receptor in male and female animals

Although ApoE levels in plasma were not detected by ELISA under our experimental conditions (see Results section 7.1.3.1), they were clearly detected in the PFC in both the control and ethanol groups (Figure 19), as expected due to its astrocyte origin.

Figure 19 shows that there were no differences between the alcohol and control groups in total ApoE levels, in the [LipidA-ApoE] bound form (Fig. 19A, B; $t_{(16)}=0.01856$, $p=0.29854$; $t_{(16)}=0.1418$, $p=0.8890$, respectively) or in ApoER2 expression (Fig. 19C; $t_{(16)}=1.082$, $p=0.2953$) in the PFC of males. Blots are represented in Fig. 19D.

Similarly, no differences were found in the total form of ApoE, the [LipidA-ApoE]-bound form and ApoER2 expression in females (Fig. 19E, F, G; $t_{(15)}=1.553$, $p=0.1413$; Mann–Whitney U = 34; $p>0.05$; $t_{(15)}=0.2900$, $p=0.7758$ n.s., respectively). Representative blots in Fig. 19H.

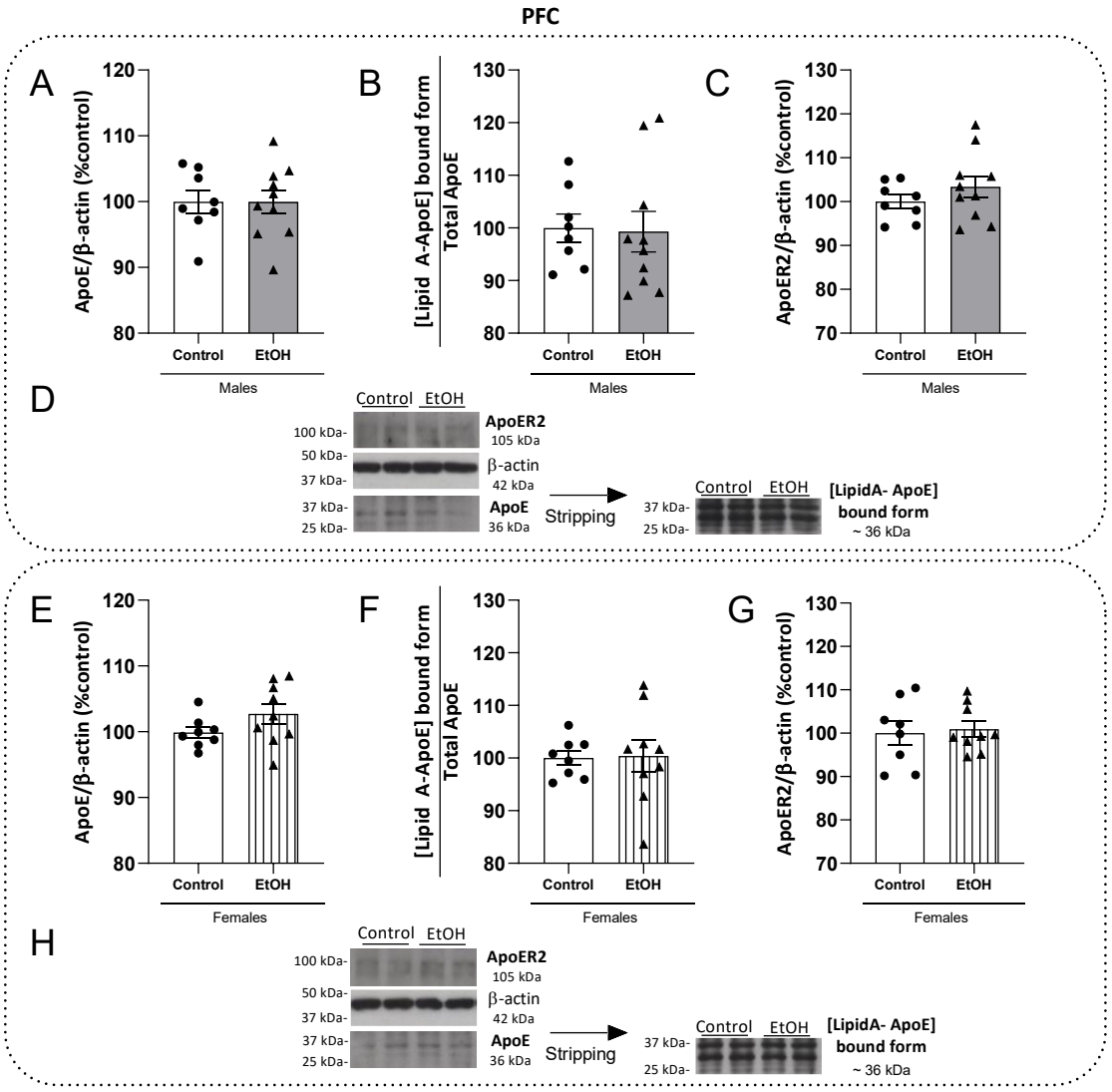



**Figure 19. Expression of ApoE and [LipidA-ApoE] by western blotting and its receptor ApoER2 in PFC.** The upper panel shows data in ethanol-treated (n=10) and control (n=8) males and the lower panel data in ethanol-treated (n=9) and control (n=8) females. **A** Expression of total ApoE in males. **B** The ratio [LipidA-ApoE] bound form in males, as indirect measurement of co-localization of proteins (Lipid A was detected at 36 kDa and normalized by total ApoE). **C** ApoER2 levels in males. **D** Representative immunoblots from the same gel of total ApoE, [LipidA-ApoE] bound form and ApoER2 in males. **E** Expression of total ApoE levels in females. **F** The ratio [LipidA-ApoE] bound form in female, as indirect measurement of co-localization of proteins (Lipid A was detected at 36 kDa and normalized by total ApoE). **G** Expression of ApoER2 levels in female rats. **H** Representative immunoblots from the same gel of total ApoE, [LipidA-ApoE] bound form and ApoER2 in females. Western blot data were normalized by β-actin and expressed as a percentage of change over controls. Results represent the mean ± S.E.M. of two technical replicates. There were no differences between groups (Student´s *t*-test, $p>0.05$). *EtOH*=ethanol-treated animals.

### 7.1.6 Expression neuroinflammatory markers in the PFC of animals exposed to alcohol binges

#### 7.1.6.1 Expression of free and bound forms of TLR4 in the PFC of male and female animals after intensive alcohol consumption

We used both western blotting and co-IP to check the binding of Lipid A to different apolipoproteins (see previous results) but also to TLR4. Figure 20A represents the total expression of TLR4 in the PFC of male rats under ethanol or control conditions. We observed that TLR4 was upregulated in ethanol-treated male rats (Fig. 20A; $t_{(16)}$=2.484, $p$=0.0244), whereas there was no significant effect in females. Notably, at the time-point of tissue extraction (3 h after the last alcohol binge), males showed higher BELs than females (Table 4), and we found a positive correlation between BELs and TLR4 expression in the PFC in male animals (Fig. 20B, $r$=0.6679, $p$<0.05) that was not found in females. The study of binding between TLR4 and Lipid A is represented in Fig. 20C for males. Data are expressed as [LipidA-TLR4] bound form normalized by the expression of total TLR4. The results indicate that the [LipidA-TLR4] bound form is decreased in male ethanol-treated animals (Fig. 20C; $t_{(16)}$=3.589, $p$=0.0025). Fig. 20D shows the representative blots for western blot analyses in males.

The bottom panel of Figure 20 shows the same parameters in female animals. As commented before, no significant effect was found in TLR4 expression in female ethanol rats compared to controls in the PFC (Fig. 20E; $t_{(15)}$=1.098, $p$=0.2897, n.s.), no correlation of TLR4 with BELs (Fig. 20F, $r$=0.3589, $p$>0.05) and no binding of TLR4-Lipid A was found in females (Fig. 20G; $t_{(15)}$=1.769, $p$=0.0971, n.s.). Fig. 20H shows representative blots in female animals.

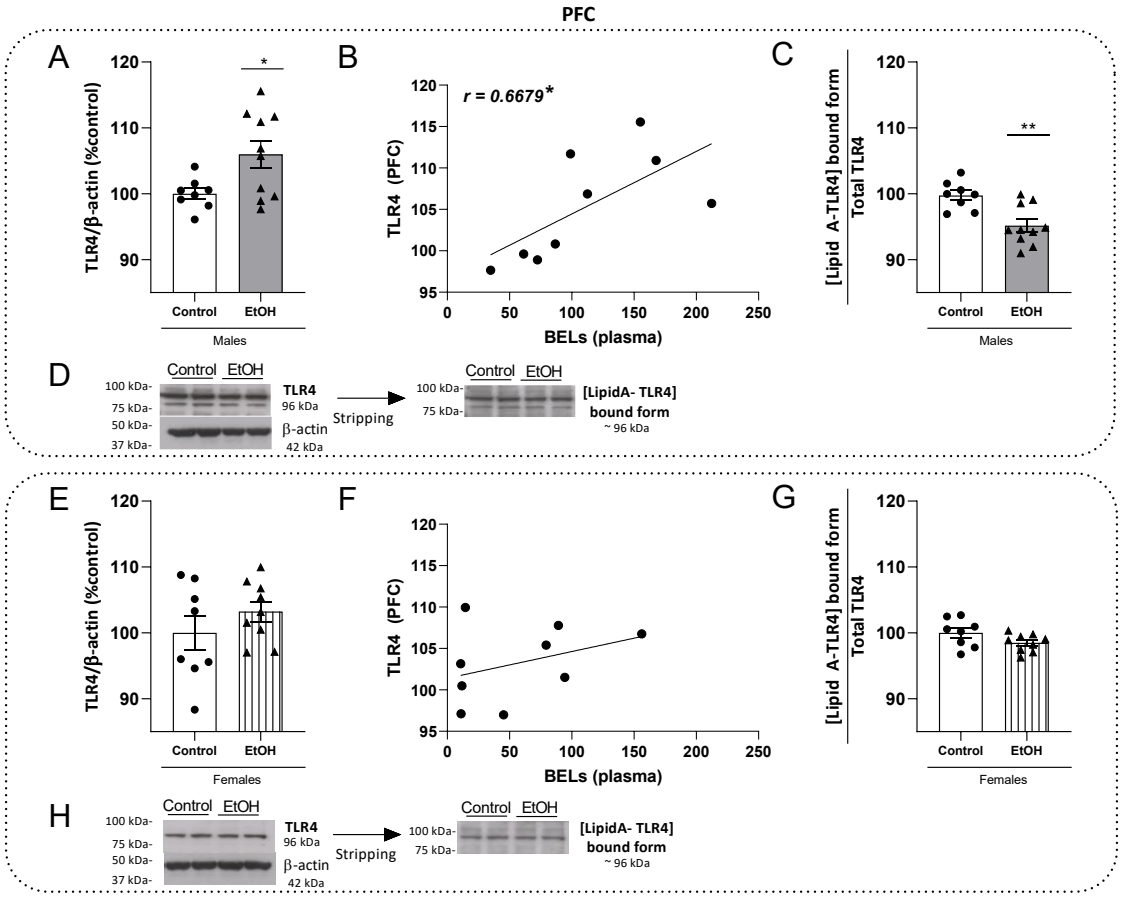



**Figure 20. TLR4 and [LipidA-TLR4] bound form expression by western blotting and TLR4-BELs linear regression in PFC.** The upper panel shows data in ethanol-treated vs control males and the lower panel data in ethanol-treated vs control females. Enzyme-linked immunosorbent assay (ELISA) data of proinflammatory cytokines in the frontal cortex levels (right-hand panel). **A** TLR4 levels were increased in ethanol-treated males. **B** Linear regression between TLR4 in PFC and BELs in plasma in males. The trend line shows the regression analyses for the ethanol-treated group. BELs in plasma were positively correlated with the TLR4 levels in PFC. **C** The ratio [LipidA-TLR4] bound form in males, as indirect measurement of co-localization of proteins, were decreased in ethanol-treated group. Lipid A was detected at 96 kDa and normalized by total TLR4. **D** Representative immunoblots of total TLR4 and [LipidA-TLR4] bound form from the same gel of males. **E** Expression of total TLR4 in females. **F** Linear regression between TLR4 in PFC and BELs in plasma females. **G** The ratio [LipidA-TLR4] bound form in females. **H** Representative immunoblots of total TLR4 and [LipidA-TLR4] bound form from the same gel in females. Western blot data were normalized by β-actin and expressed as a percentage of change over controls. Results represent the mean ± S.E.M. of two technical replicates. Differences from control group: *$p$<0.05, **$p$<0.01 (Student's $t$-test or Pearson's coefficient correlation $r$). *EtOH*=ethanol-treated animals; *BELs*=blood ethanol levels. *TLR4*=Toll-like receptor 4. *PFC*=prefrontal cortex; *EtOH*=ethanol-treated animals.

### 7.1.6.2 Expression of pro-inflammatory cytokines in the PFC of male and female animals after intensive alcohol consumption

Figure 21 represents the expression of pro-inflammatory cytokines, IL-1β and TNF-α, in the PFC of males and females under control or ethanol conditions. Results were analysed using 2-way ANOVA, since samples could be loaded together, in order to study differences between the four groups, including sexual differences.

IL-1β showed a tendency to increase in the ethanol-treated groups versus control groups, although it did not reach statistical significance (Fig. 21A; 2-way ANOVA, overall alcohol effect: $F_{(1, 29)} = 3.483$, $p$=0.0721), disregarding of sex ($F_{(1, 29)} = 1.918$; $p$=0.1766; no interaction $F_{(1, 29)} = 0.04833$; $p$=0.8275).

Regarding TNF-α, we did not find significant changes in PFC after ethanol administrations (Fig. 21B; 2-way ANOVA, overall alcohol effect: $F_{(1, 30)} = 0.3470$, $p$=0.5602), or sex effects ($F_{(1, 30)} = 0.09687$; $p$=0.7578; no interaction $F_{(1, 30)} = 0.02734$; $p$=0.8698).

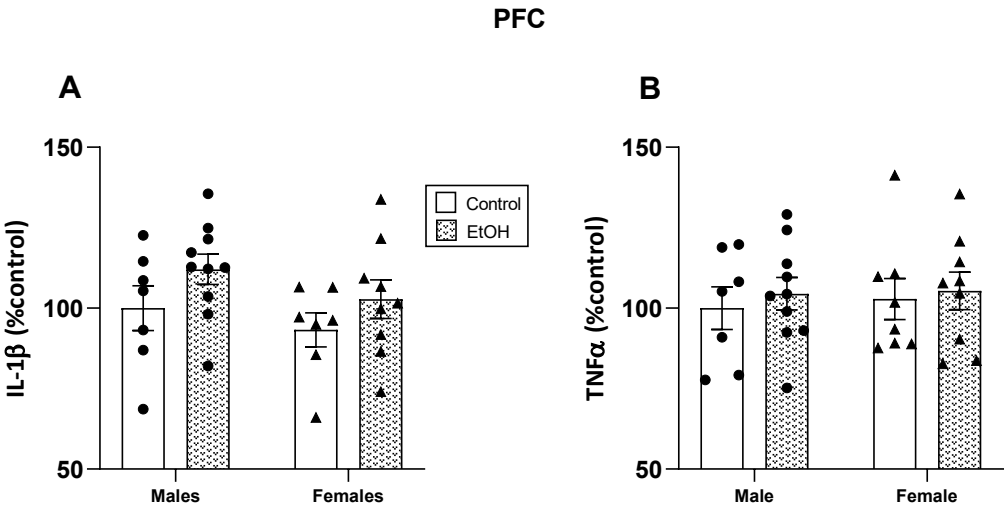



**Figure 21. Expression of pro-inflammatory cytokines in the PFC of male and female ethanol-treated and control animals**. **A** IL-1β expression in the PFC. **B** TNF-α expression in the PFC. All data are expressed as mean ± S.E.M. Statistical analysis: 2-way ANOVA: non-significant (n.s.). *EtOH*=ethanol-treated animals. *PFC*=prefrontal cortex; *EtOH*=ethanol-treated animals.

### 7.1.7 Analysing the contribution of cerebral blood flow to the presence of bacterial products within the brain

We have shown the presence of bacterial components in their free form or bound to apolipoproteins within brain areas affected by alcohol, such as the PFC, in previous sections. Although it was not a fundamental objective of the present thesis, we performed a pilot experiment to ascertain the influence of blood flow within the brain in the changes observed. In this pilot study we compared perfused (blood removed) and non-perfused female control rats to study the presence of free Lipid A and some of its aggregates, for example ApoAI or TLR4, in physiological conditions.

Figure 22 shows that free Lipid A levels were lower when the blood was removed from the animals compared with non-perfused rats ($t_{(13)}$ =4.020; $p$=0.0015), according to the well-known peripheral origin of LPS. However, levels of free Lipid A were still detectable in perfused animals (Fig. 22A). Interestingly, we did not observe differences in the LipidA-TLR4 aggregates (Fig. 22C,D. Mann–Whitney, U=24, $p > 0.05$; $t_{(14)}$ =1.022; $p$=0.3239) or LipidA-ApoAI bound forms (Fig. 22E,F. $t_{(14)}$ =0.2082; $p$=0.8381; $t_{(14)}$ =0.7757; $p$=0.4508) in the PFC of perfused versus non-perfused animals. These results suggest that an important percentage of Lipid A is retained in the blood vessels within the PFC but that the aggregated forms with receptors or apolipoproteins are present within the cerebral blood vessel structures or in the brain parenchyma, as suggested before by other authors showing co-localization with immunohistochemistry procedures (Vargas-Caraveo et al., 2017).

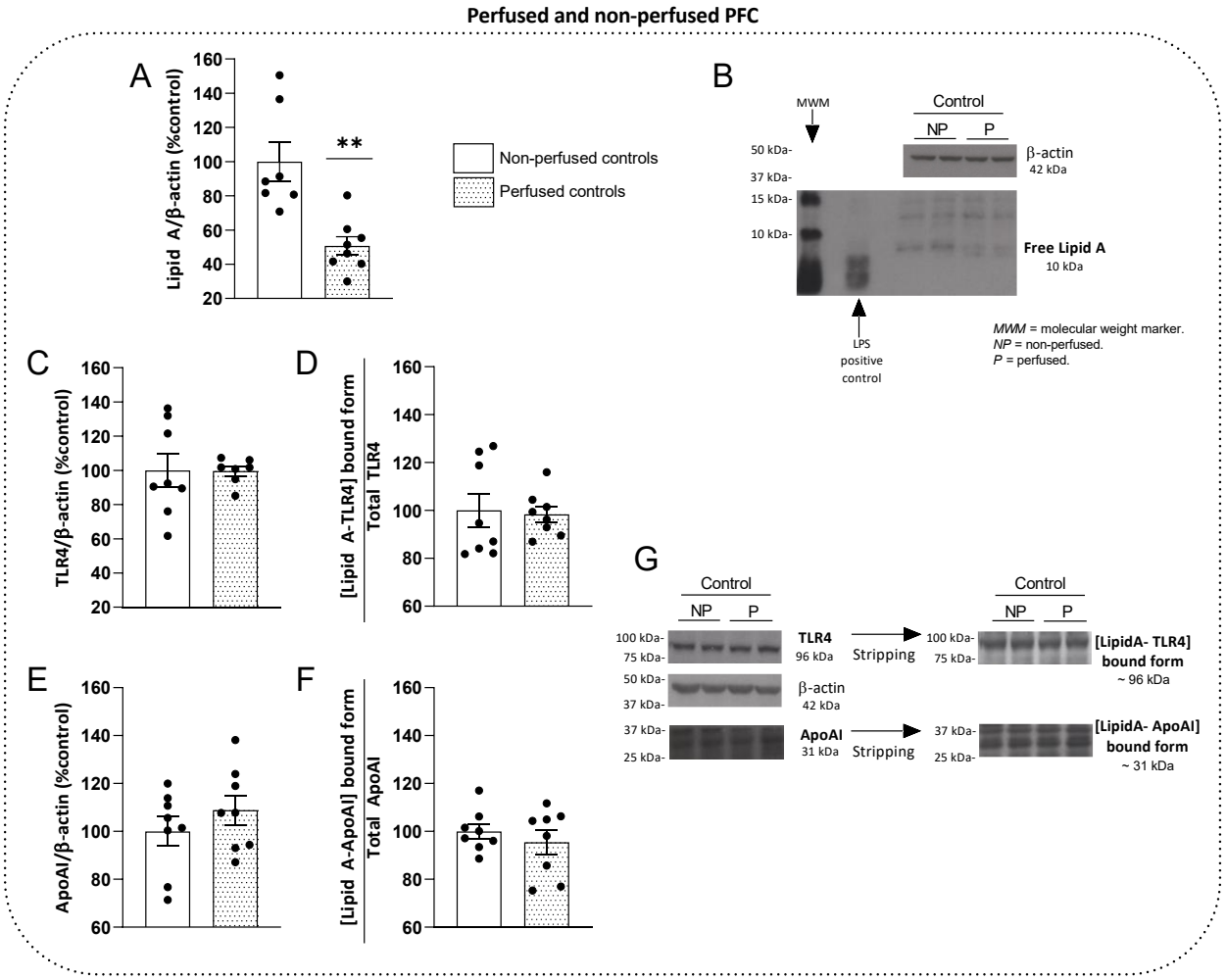



**Figure 22. Presence of free Lipid A and [LipidA-protein] aggregates in PFC of perfused and non-perfused female controls. A** Expression of free Lipid A. **B** Representative immunoblots of Lipid A. **C** TLR4 levels. **D** Ratio [LipidA-TLR4] bound form. Lipid A was detected at 96 kDa and normalized by total TLR4. **E** ApoAI levels. **F** Ratio [LipidA-ApoAI] bound form Lipid A was detected at 31 kDa and normalized by total ApoAI. **G** Representative immunoblots of total TLR4, total ApoAI, [LipidA-TLR4] and [LipidA-ApoAI] bound forms from the same gel. Western blot data were normalized by β-actin and expressed as a percentage of change over non-perfused controls. Results represent the mean ± S.E.M. of two technical replicates. Differences between groups: $^{**}p<0.01$ (Student's *t*-test). *MWM*=molecular weight marker; *NP*=non-perfused controls; *P*=perfused controls; *EtOH*=ethanol-treated animals.

### 7.1.8 Expression of apolipoproteins and its receptors and formation of aggregates with bacterial products in the cerebellum of animals exposed to alcohol binges

The whole study was repeated in the cerebellum, both in the vermis and in the cerebellar hemispheres (Hcb) (See Supplemental López-Valencia et al., 2024). Results in vermis showed no significant differences between groups (data not shown). Results in Hcb are summarized in this section.

### 7.1.8.1 Presence of LPS components (Lipid A and Core) and expression of TLR4 in the Hcb of alcohol-intoxicated males and females and control animals

Lipid A, core and TLR4 expressions were measured in the cerebellar hemisphere by western blotting, but no significant differences were found in any of them (Figure 23) between ethanol and control groups neither in males (Fig. 23A,B,C; $t_{(16)}$=1.529, $p$=0.1457; $t_{(16)}$=1.410, $p$=0.1778; $t_{(16)}$=0.6408, $p$=0.5307, respectively) nor females (Fig. 23E,F,G; $t_{(15)}$ =1.958, $p$=0.0691; $t_{(15)}$=0.5886, $p$=0.5649; $t_{(15)}$=1.187, $p$=0.2538, respectively). However, Lipid A levels in the female ethanol group showed a tendency to decrease (Student´s $t$-test near of significance, $p$=0.0691) that is contrary to the tendency observed in males.

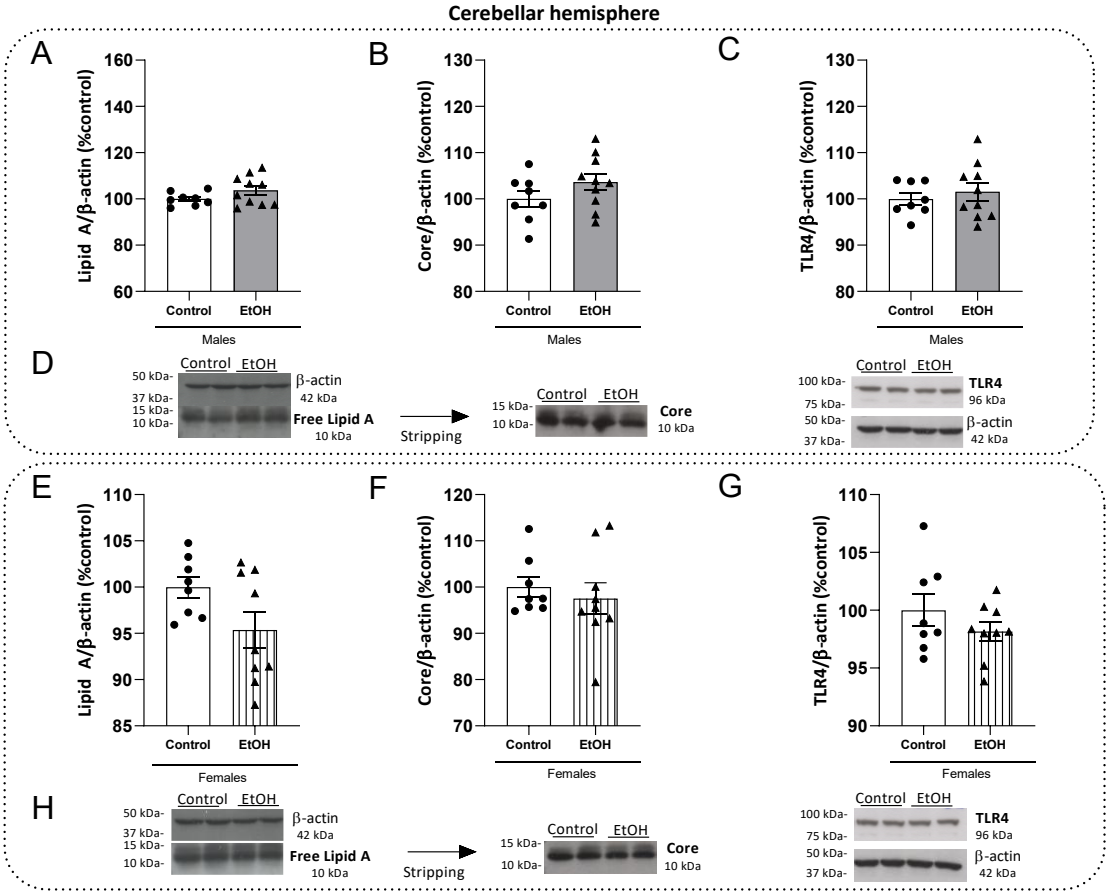



**Figure 23. Detection of Lipid A and core and TLR4 expression in cerebellar hemisphere by western blotting**. **A** Expression of Lipid A in males. **B** Expression of the Core element of LPS in males. **C** Expression of TLR4 in males. **D** Representative blots. **E** Expression of Lipid A levels in females. **F** Expression of Core in females. **G** Expression of TLR4 levels in female rats. **H** Representative blots. Western blot data were normalized by β-actin and expressed as a percentage of change over controls. Results represent the mean ± S.E.M. of two technical replicates. No differences were observed between groups (trend in Lipid A in females (E): difference from control group $p$=0.0691; Student's $t$-test). *EtOH*=ethanol-treated animals.

### 7.1.8.2 Expression of free and bound forms of Apolipoprotein AI in the Hcb and the SR-BI receptor in male and female animals

Regarding expression and binding to apolipoproteins in the cerebellar hemispheres, ApoAI was detected in this brain region, but no differences were found in its total or bound form with Lipid A (Fig. 24A,B; $t_{(15)}$=0.5670, $p$=0.5791; $t_{(16)}$=0.7446, $p$=0.4673, respectively) or in the SR-BI expression (Fig. 24C, $t_{(16)}$=0.1125, $p$=0.9118) in males Hcb.

Similarly, no differences were found in Lipid A, the [LipidA-ApoAI] bound form and the SR-BI expression in females (Fig. 24E,F,G; $t_{(15)}$=0.1165, $p$=0.9088; $t_{(15)}$=0.8680, $p$=0.3991; $t_{(15)}$=1.012, $p$=0.3274, respectively).

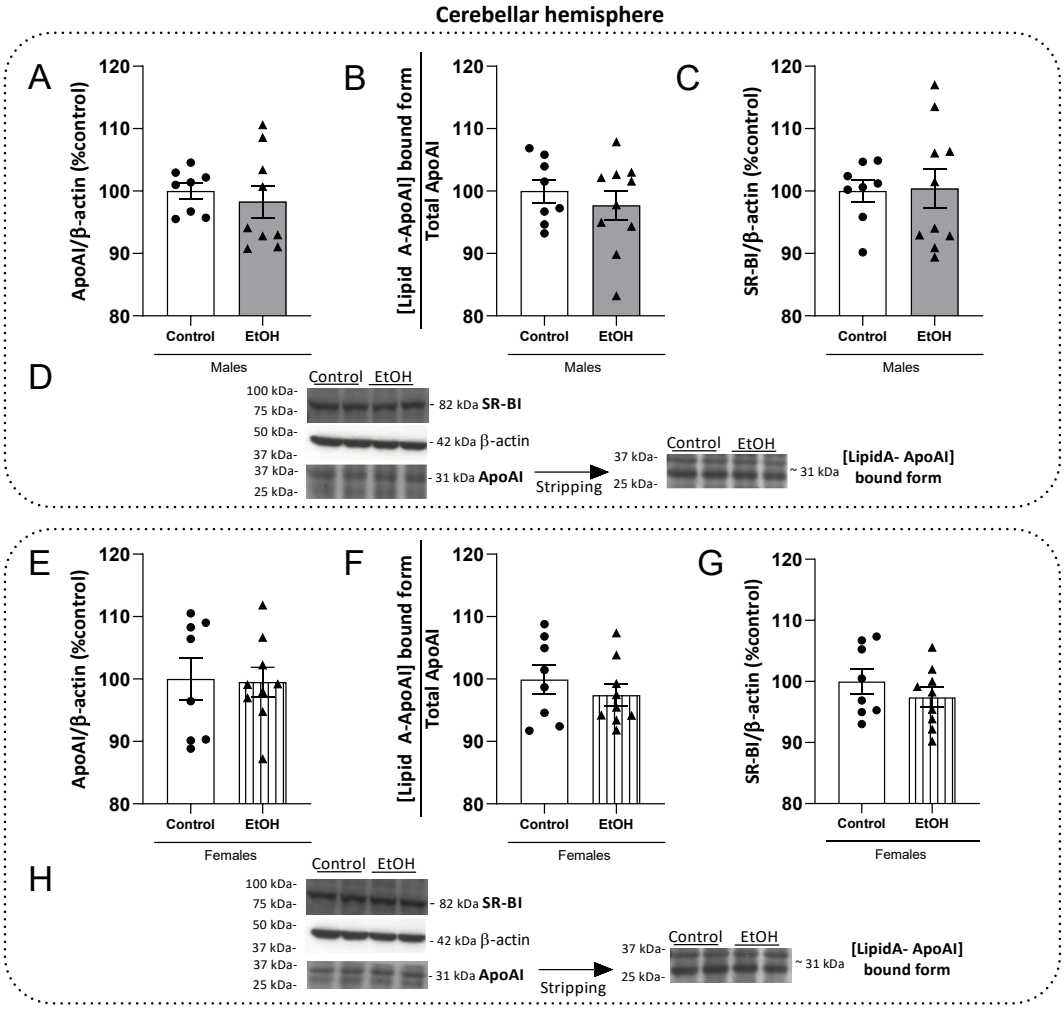



**Figure 24. Expression of ApoAI, [LipidA-ApoAI] bound form and SR-BI in cerebellar hemisphere**. **A** Total ApoAI in males. **B** [LipidA-ApoAI] bound form in males. **C** Expression of SR-BI in males. **D** Representative blots. **E** Total ApoAI levels in females. **F** [LipidA-ApoAI] bound form in females. **G** Expression of SR-BI levels in females. **H** Representative blots. Western blot data were normalized by β-actin and expressed as a percentage of change over controls. Results represent the mean ± S.E.M. of two technical replicates. No differences were observed between groups (Student's *t*-test). *EtOH*=ethanol-treated animals.

### 7.1.8.3 Expression of the free and bound forms of Apolipoprotein B in the Hcb and the LDL receptor in male and female animals

Similarly, no differences were detected in total ApoB, [LipidA-ApoB] bound form or LDLr expression in males (Fig. 25A, B, C; $t_{(16)}$=1.226, $p$=0.2378; Mann-Whitney U=38, $p$>0.05, n.s.; $t_{(16)}$=1.822, $p$=0.0873, respectively) or females (Fig. 25E, F, G; $t_{(15)}$=1.049, $p$=0.3107; $t_{(15)}$=0.4311, $p$=0.6725; $t_{(15)}$=0.09363, $p$=0.9266, respectively).

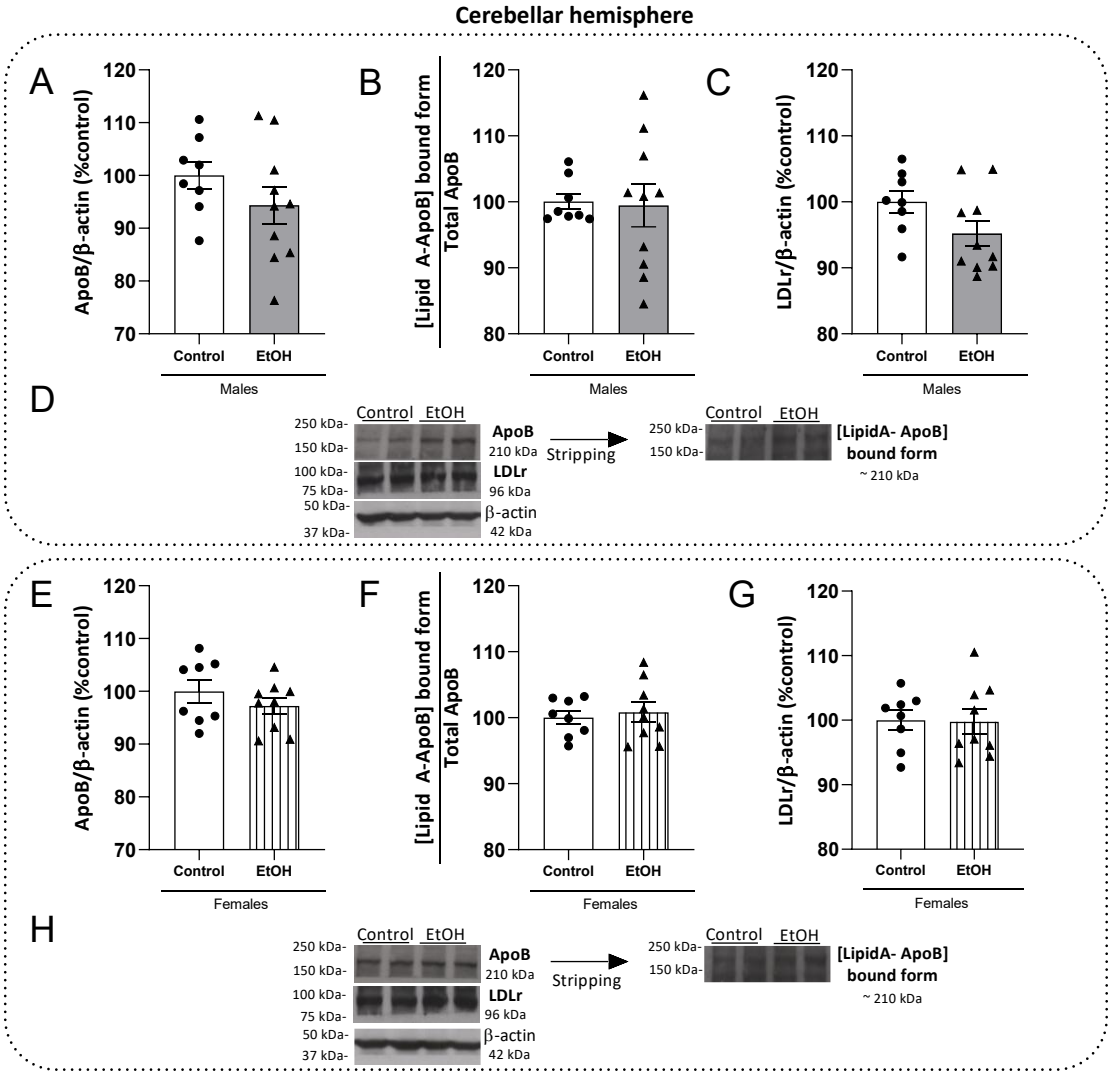



**Figure 25. Expression of ApoB, [LipidA-ApoB] bound form and LDLr in cerebellar hemisphere**. **A** Total ApoB in males. **B** [LipidA-ApoB] bound form in males. **C** LDLr in males. **D** Representative blots. **E** Total ApoB in females. **F** [LipidA-ApoB] bound form in females. **G** LDLr levels in females. **H** Representative blots. Western blot data were normalized by β-actin and expressed as a percentage of change over controls. Results represent the mean ± S.E.M. of two technical replicates. No differences were observed between groups (Student's *t*-test). *EtOH*=ethanol-treated animals.

### 7.1.8.4 Expression of the free and bound forms of Apolipoprotein E in the Hcb and the ApoER2 receptor in male and female animals

ApoE was also detected in the cerebellar hemisphere in both groups with no significant differences in its total or bound form in males (Fig. 26A,B; $t_{(16)}$=1.517, $p$=0.1487; $t_{(16)}$=0.9982, $p$=0.3330, respectively). Interestingly, levels of ApoER2 in Hcb were upregulated in the ethanol group in males (Fig. 26C; Mann-Whitney U=10, $p$<0.05).

Similarly, no differences were found in ApoE in its total or bound form in females (Fig. 26E,F; $t_{(14)}$=0.6143, $p$=0.5488; $t_{(13)}$=0.6697, $p$=0.5147, respectively). Contrary to what it was observed in males, ApoER2 was downregulated in the ethanol group (Fig. 26G; $t_{(14)}$=3.133, $p$=0.0073).

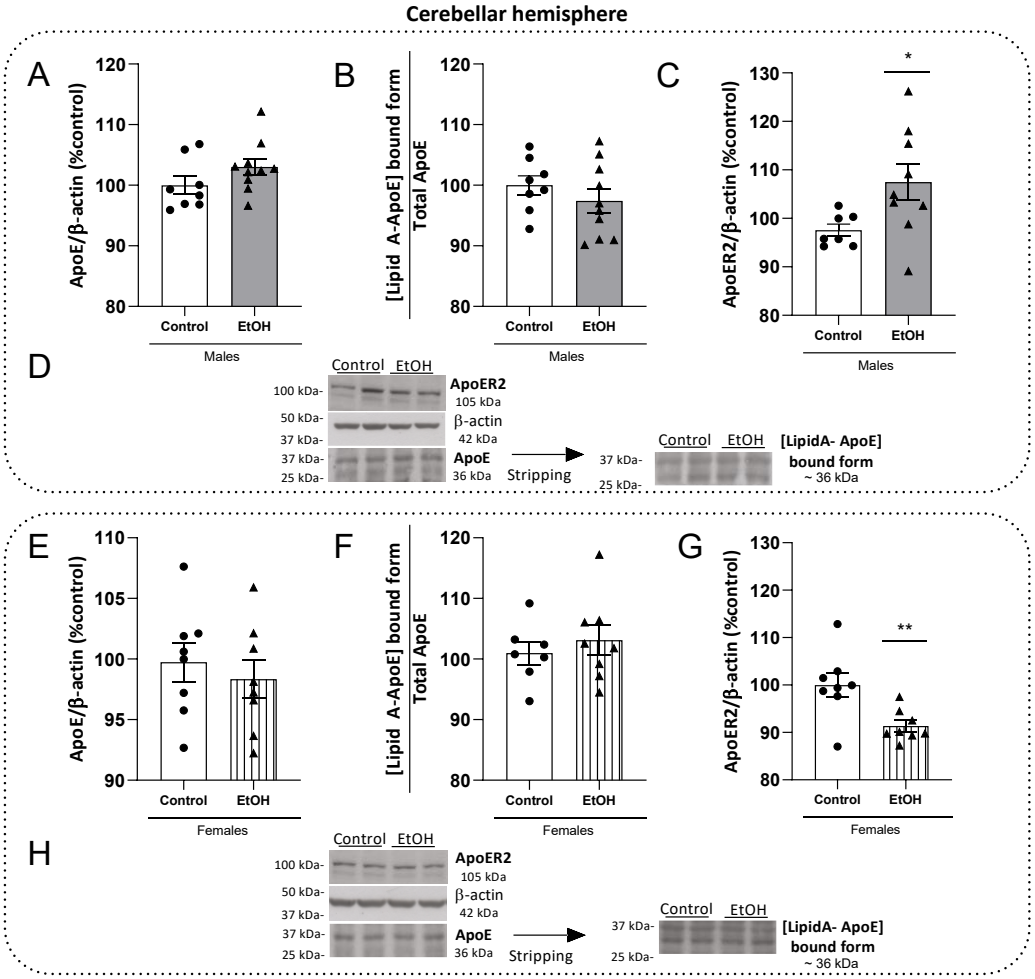



**Figure 26. Expression of ApoE, [LipidA-ApoE] and ApoER2 in cerebellar hemisphere**. **A** Total ApoE in males. **B** [LipidA-ApoE] bound form in males. **C** ApoER2 in males. **D** Representative blots. **E** Total ApoE levels in females. **F** [LipidA-ApoE] bound form in females. **G** ApoER2 levels in females. **H** Representative blots. Western blot data were normalized by β-actin and expressed as a percentage of change over controls. Results represent the mean ± S.E.M. of two technical replicates. Different from control group: *$p$<0.05, **$p$<0.01 (Mann-Whitney (C) or Student's $t$-test (G)). *EtOH*=ethanol-treated animals.

## 7.2 STUDY II: THE EFFECTS OF APOLIPOPROTEIN AI POTENTIATION ON THE NEUROINFLAMMATORY AND NEUROBEHAVIOURAL RESPONSES AFTER INTENSIVE ALCOHOL CONSUMPTION

In the previous study, sex differences were found regarding the apolipoproteins that binds Lipid A under alcohol intoxication conditions. Whereas Lipid A is aggregated with ApoAI in the PFC of female rats, this bacterial component is bound to ApoB in males. A subsequent strategy to study the implications of these bindings on behaviour would be to enhance the biological effects of these Apos. Thus, it would be of interest to enhance ApoAI in females and ApoB in males.

We initiate these studies with females, since female animals are a current focus of scientific interest, due to the lack of research conducted on them in comparison to males. Thus, the results presented in this section detail the efforts to mimic ApoAI activity in female rats exposed to alcohol binges. In order to do that, two distinct strategies for enhancing ApoAI were followed: the administration of HDL, the lipoprotein with major content in ApoAI, and the use of an ApoAI mimetic peptide.

### 7.2.1 Effects of HDL pre-treatment in females exposed to ABD protocol and control animals

Administration of HDL is an unspecific way to potentiate ApoAI, since this lipoprotein is composed of 70% of ApoAI (Dominiczak & Caslake, 2011; Feingold, 2021; Phillips, 2013). In this section we describe the effects of HDL administration in ethanol-induced LPS rise and neuroinflammation at early abstinence (3h after last binge) and ethanol-induced neurobehavioral responses starting at 3h of abstinence in female animals.

#### 7.2.1.1 Bacterial products after HDL pre-treatment in alcohol-intoxicated and control rats: plasma and brain LPS

We checked the expression of LPS in plasma and brain 3h after the last binge administration. Results, analysing by 2-way ANOVA (factors: alcohol/water & HDL/vehicle), revealed no effect of alcohol on LPS plasma levels at that time of abstinence ($F_{(1,20)}$=1.409, $p$=0.2491, n.s.). However, higher levels of plasma LPS were detected in HDL-pretreated animals, disregarding of the experimental group (alcohol/control) (Fig. 27; HDL main effect; $F_{(1,20)}$=6.570, $p$=0.0185). No interaction between factors was found ($F_{(1,20)}$=0.4393, $p$=0.5150, respectively).

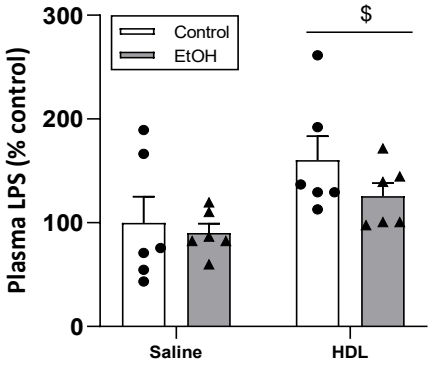



**Figure 27. LPS levels in the plasma of HDL-pretreated female animals in ABD and control groups 3h after the last ethanol administration.** Plasma levels detected by ELISA and expressed as a percentage of controls. Basal levels of plasma LPS in saline-pretreated control animals: 1.097±0.28 EU/mL. Data represents the mean ± S.E.M. HDL main effect: $^{\$}p<0.05$ (2-way ANOVA). *LPS*: Lipopolysaccharide; *EtOH*=ethanol-treated animals.

We also checked the possible presence of LPS within the brain at that time. We were able to detect the presence of minimal LPS in the PFC of control female animals. The ABD procedure did not significantly alter the brain expression of LPS (Fig. 28; $F_{(1,21)}=1.984$, $p=0.1736$), in spite some animals showed 2 or 2.5 fold increases versus controls (Fig. 28). HDL pre-treatment did not alter LPS brain expression (Fig. 28; $F_{(1,21)}=0.2023$, $p=0.6575$, respectively) and no interaction between factors was found ($F_{(1,21)}=0.0008278$, $p=0.9773$).

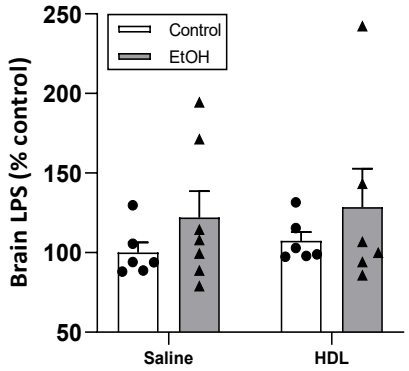



**Figure 28. LPS levels in the FC of HDL-pretreated female animals in ABD and control groups 3h after the last ethanol administration.** Brain levels detected by LAL assay and expressed as a percentage of controls. Basal levels of brain LPS in saline-pretreated control animals: 1.80±0.12 EU/mL. Data represents the mean ± S.E.M. *LPS*=Lipopolysaccharide; *EtOH*=ethanol-treated animals.

#### 7.2.1.2 Effects of HDL pre-treatment on the neuroinflammation in the PFC in alcohol-intoxicated and control rats

As mentioned in the Methods section, these results were obtained during my international stay (December 2023-April 2024) at the University of Oxford (Department of Pharmacology), in

collaboration with Prof. Daniel Anthony's group. In brain samples obtained from our ABD experiments in the Laboratory of Psychobiology of the Complutense University of Madrid, we explored whether HDL pre-treatment could promote the gene expression of some pro-inflammatory markers, such as cytokines, or the innate immune system receptor TLR4. The levels of the different mediators were measured 3h after the last ethanol binge administration (see method section 6.9).

It is important to note that the effects of this IAC protocol are largely unknown in females, since most of the previous studies were done in male animals. Previous studies in our laboratory have shown that this protocol of ABD in males can promote the gene expression of some cytokines, such as IL-1β and TNF-α, and an upregulation of the TLR4 expression in the PFC (Antón et al., 2017). In this study, a similar ABD protocol (modified from Antón et al., 2017) did not alter main markers of neuroinflammation in the PFC of females, such as HMGB1, IL-1β or TNF-α (Fig. 29B,E,F; $F_{(1,21)}$=0.02376, $p$=0.8790; $F_{(1,20)}$=0.4370, $p$=0.5161; $F_{(1,21)}$=0.2944, $p$=0.5931, respectively), although some tendencies were observed for TLR4 and IL-6 (Fig. 29A,D; $F_{(1,21)}$=3.307, $p$=0.0833; $F_{(1,20)}$=3.231, $p$=0.0874, respectively). Alcohol did affect the expression of CXCL1 in the limit of significance and disregarding of the HDL-pretreatment (Fig. 29C; $F_{(1,19)}$=4.206, $p$=0.0543).

Analysing the influence of HDL-pretreatment in both groups, we found a higher expression of TLR4 mRNA in the PFC of both ethanol and control groups (HDL main effect. Fig. 29A. 2-way ANOVA. $F_{(1,21)}$=4.421, $p$=0.0477). Similarly, we observed a tendency to increase in the HMGB1 mRNA expression in both HDL-pretreated groups, being the HDL main effect on the limit of statistical significance (Fig. 29B; $F_{(1,21)}$=4.228, $p$=0.0524). HDL-pretreatment also induced a main effect in CXCL1 mRNA (Fig. 29C; $F_{(1,19)}$=7.678, $p$=0.0122) and no interaction between factors were found in any of them ($F_{(1,21)}$=0.002572, $p$=0.9600; $F_{(1,21)}$=0.01631, $p$=0.8996; $F_{(1,19)}$=0.3127, $p$=0.5825, respectively). No differences were found in IL-6, IL-1β or TNF-α mRNA expression due to HDL pre-treatment, although a trend can be observed for this latest (Fig. 29D,E,F; $F_{(1,20)}$=1.221, $p$=0.2823; $F_{(1,20)}$=0.8501, $p$=0.3675; $F_{(1,21)}$=3.678, $p$=0.0688, respectively). No interaction between factors were observed in any of them ($F_{(1,20)}$=0.2194, $p$=0.6446; $F_{(1,20)}$=0.1925, $p$=0.6655; $F_{(1,21)}$=0.1290, $p$=0.7231, respectively), suggesting that both alcohol and HDL had independent effects.

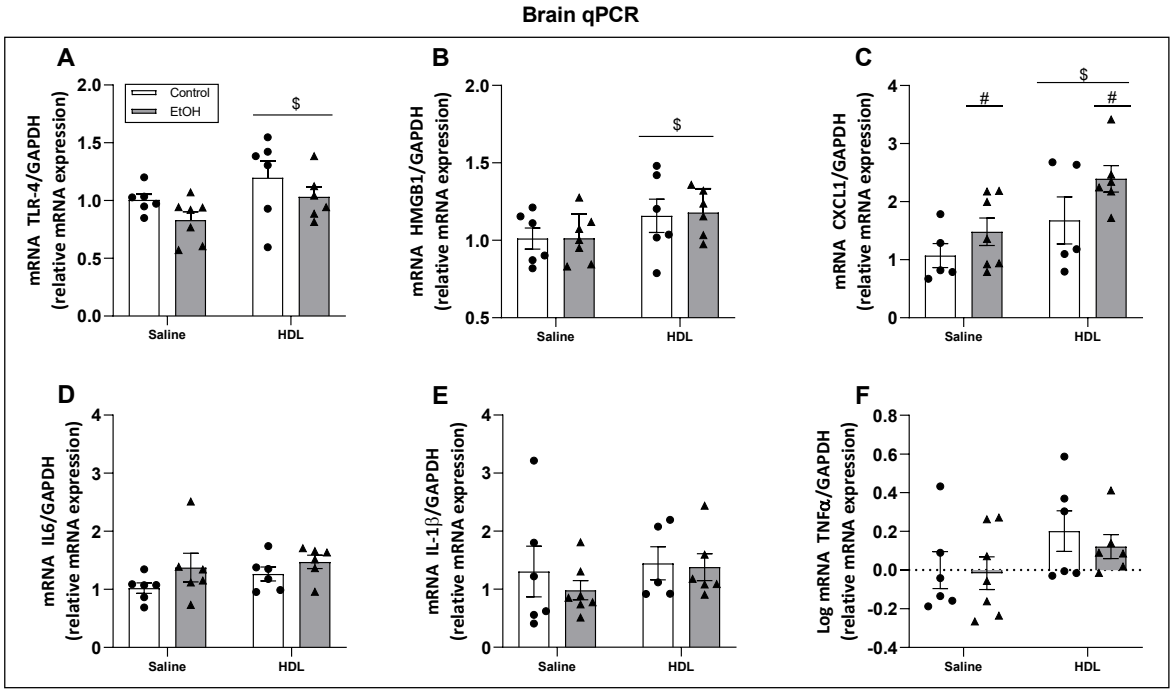



**Figure 29. Effects of HDL pre-treatment on frontal cortex on main neuroinflammatory parameters in female rats exposed to alcohol binges and in control animals.** mRNA relative levels of **A** TLR4, **B** HMGB1, **C** CXCL1, **D** IL6, **E** IL-1β and **F** TNF-α. mRNA levels were normalized by GAPDH. Parameters were measured 3h after the last ethanol binge. 2-way ANOVA: EtOH main effect: $^{\#}p < 0.05$; HDL main effect $^{\$}p < 0.05$. *EtOH*=ethanol-treated animals.

This pilot study suggests that HDL pre-treatment may induce a proinflammatory profile in control and ABD animals by itself and with independence of the alcohol effects.

#### 7.2.1.3 Effects of HDL pre-treatment on the neurobehavioural alterations in animals exposed to ABD during early abstinence and control animals

This study was carried out in an independent experiment, using similar methodology (see method section, figure 7B), to characterize the psychopharmacological effects of HDL pretreatment in alcohol and control female rats.

It is well known that ABD induces behavioural alterations during abstinence related to a strong inflammatory response (Marco et al., 2017; Montesinos, Alfonso-Loeches, et al., 2016; Pascual et al., 2017). In previous studies we have shown that ABD induces also aggregation of apolipoproteins with bacterial products, which are the main source of alcohol-induced inflammation. The effects of apolipoprotein administration at the behavioural level have not been

tested. Specifically, as explained in previous chapters of this Doctoral Thesis, we showed that Lipid A (the active component of LPS) is bound to ApoAI in the PFC of females exposed to ethanol binges. The implications of this binding on behaviour and neuroinflammation remain unclear. Thus, in the following study we explore the effects of HDL administration as a pre-treatment in our ABD model and in control female animals. Results are summarized in the following lines, according to behavioural tests related to emotional or cognitive assessment.

## EMOTIONAL ASSESSMENT

### Anxiety-like behaviour

Contrary to **anxiety-like behaviour** results found in males (Antón et al., 2017), in female animals we did not observe anxiety symptoms 12h after the last ethanol binges in the EPM either in the percentage of entries in the open arms (Fig. 30A; $F_{(1,32)}$=0.9238, $p$=0.3437) or in the time spent in these open arms (Fig. 30; $F_{(1,32)}$=0.006545, $p$=0.9360).

Similarly, no main effects of HDL pre-treatment were found either in open arm entries (Fig. 30A; $F_{(1,32)}$=1.527, $p$=0.2255) or open time (Fig. 30B; $F_{(1,32)}$=0.1829, $p$=0.6718). In addition, no interaction between factors were observed ($F_{(1,32)}$=0.8232, $p$=0.3710; $F_{(1,32)}$=0.2951, $p$=0.5908, respectively).

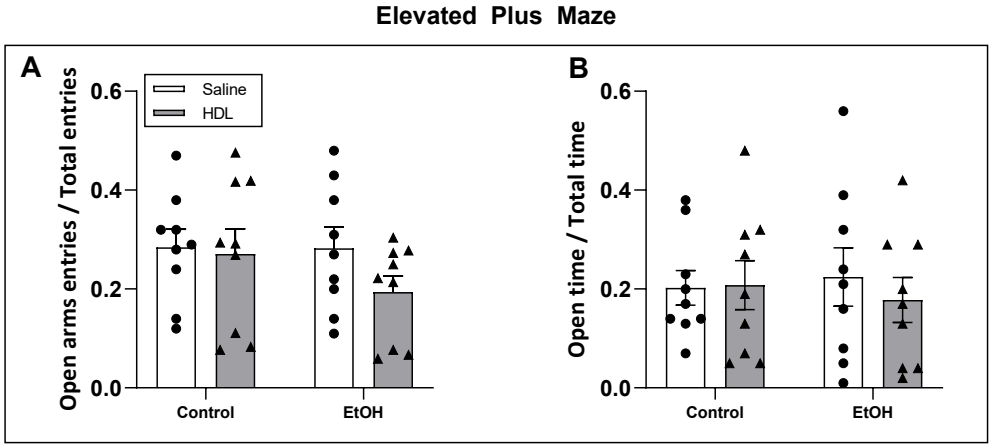



**Figure 30. Effects of HDL pre-treatment on anxiety-like behaviour in the elevated plus maze in female rats. A** Number of open arm entries over the total entries into any arm. **B** Time spent in the open arms over the total time spent in both arms. Results represent the mean ± S.E.M. No significant differences were found by 2-way ANOVA (factors: experimental group x pre-treatment). HDL was injected i.p. 15 min previous each alcohol binge; control animals received saline as a vehicle. Animals were assessed 12h after the last alcohol binge. *EtOH*=ethanol-treated animals.

Although we did not observe anxiety-like behaviour in the EPM, female rats did show an anxiety phenotype after ABD in the OFT. We observed an ethanol main effect with a decrease in the time spent in the centre of the arena (Fig. 31A; $F_{(1,32)}$=12.97, $p$=0.0011) and in the number of centre entries (Fig. 31B; $F_{(1,32)}$=19.03, $p$=0.0001), grid crossings (Fig. 31C; $F_{(1,32)}$=21.68, $p$<0.0001) and rears (Fig. 31D; $F_{(1,31)}$=9.963, $p$=0.0035), indicative of anxiety-like behaviour.

Regarding HDL pretreatment, we did not observe HDL main effects in the time spent in the centre of the arena (Fig. 31A; $F_{(1,32)}$=1.768, $p$=0.1930), centre entries (Fig. 31B; $F_{(1,32)}$=0.4025, $p$=0.5303) or grid crossings (Fig. 31C; $F_{(1,32)}$=0.01120, $p$=0.9164). However, we observed a decrease in the number of rears with an HDL main effect on the limit of statistical significance (Fig. 31D; $F_{(1,31)}$=3.824, $p$=0.0596), indicative of anxiety-like behaviour. No interaction between factors were observed in any of these parameters ($F_{(1,32)}$=0.008642, $p$=0.9265; $F_{(1,32)}$=0.3082, $p$=0.5827; $F_{(1,32)}$=0.1183, $p$=0.7331; $F_{(1,31)}$=2.899, $p$=0.0986, respectively).

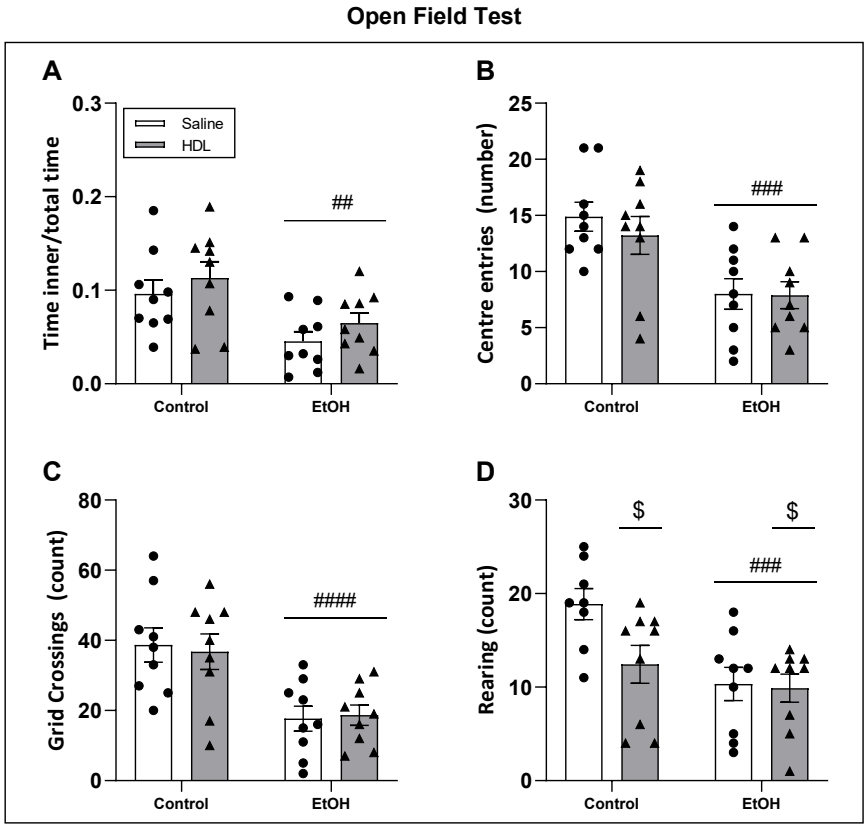



**Figure 31. Effects of HDL pre-treatment on anxiety-like behaviour in the open field test in female rats. A** Time spent in the inner zone (s). **B** Number of entries to the centre zone. **C** Number of grid crossings. **D** Number of rears, standing on hind legs. HDL was injected i.p. 15 min previous each alcohol binge; control animals received saline as a vehicle. Results represent the mean ± S.E.M. Two-way ANOVA: EtOH main effect: $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$; HDL main effect $^{\$}p < 0.05$. *EtOH*=ethanol-treated animals.

### Depressive-like behaviour and anhedonia

**Depressive-like behaviour** was observed in female animals exposed to ABD in the Forced Swimming Test. Specifically, we found an ethanol main effect in the climbing and immobility times (Fig. 32B,C; $F_{(1,32)}$=7.664, $p$=0.0093, $F_{(1,32)}$=9.722, $p$=0.0038, respectively), whereas no main effect of ethanol was found on swimming time and latency to immobility, although a tendency to decrease was observed for the latter (Fig. 32A,D; $F_{(1,31)}$=0.005696, $p$=0.9403 $F_{(1,32)}$=3.171, $p$=0.0845).

In HDL-pretreated animals, we did not observe a main effect of HDL on either swimming or climbing time (Fig. 32A,B; $F_{(1,31)}$=0.1445, $p$=0.7064; $F_{(1,32)}$=1.172, $p$=0.2872, respectively) nor on immobility or latency to immobility (Fig. 32C,D; $F_{(1,32)}$=1.474, $p$=0.2336; $F_{(1,32)}$=1.837, $p$=0.1848, respectively).

However, climbing and immobility times showed an interaction between factors (ethanol and HDL) ($F_{(1,32)}$=7.202, $p$=0.0114; $F_{(1,32)}$=10.56, $p$=0.0027, respectively). *Post hoc* comparisons revealed a decreased in the climbing time in ethanol-treated rats in comparison to controls ($p$<0.01) and an increased in the immobility time in females of the ethanol group vs control group ($p$<0.001) and between control groups (saline vs. HDL, $p$ < 0.05), indicative of depressive-like behaviour.

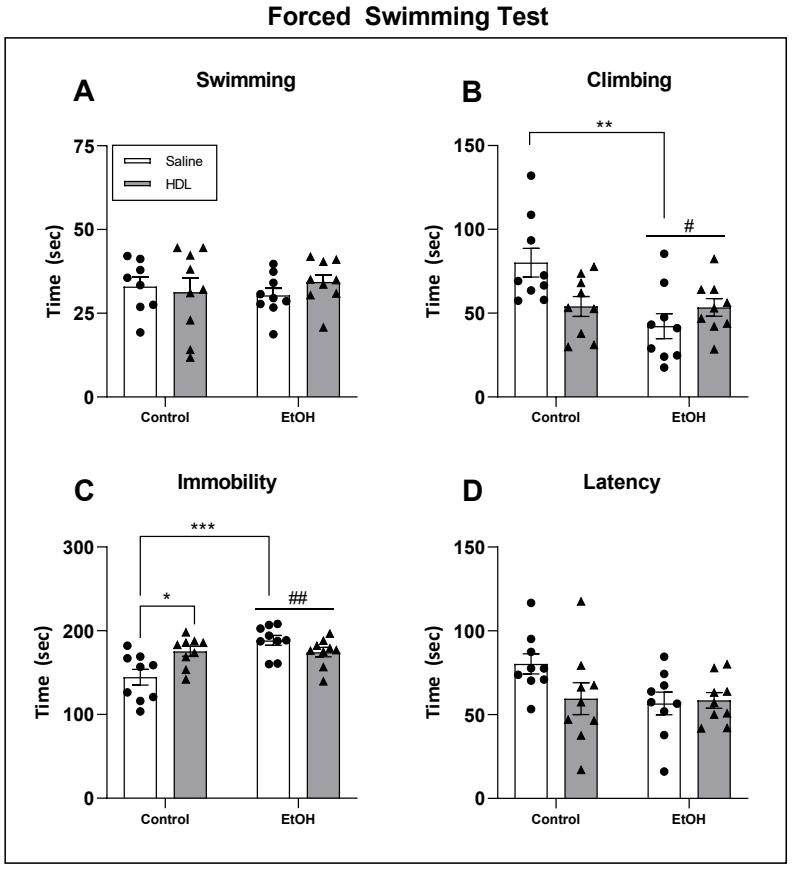


**Figure 32. Forced Swimming test for depressive-like behaviour in female rats exposed to ethanol binges and pretreated with HDL and in control animals**. **A** Swimming time. **B** Climbing time. **C** Immobility time. **D** Latency to immobility time. Two-way ANOVA: EtOH main effect: $^{\#\#}p < 0.01$; interaction followed by Bonferroni *post hoc* test: different from control (water + saline) group $^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. *EtOH*=ethanol-treated animals.

**Anhedonia**, a core symptom of depression, was measured with the SPT starting 36h after the last ethanol binge. Figure 33 shows the cumulative saccharin consumption at different measurement times along alcohol abstinence. Repeated-measures 3-way ANOVA (independent factors: time x EtOH x HDL) showed that ethanol administration had no effect on this motivational test in female rats at the time-points of testing ($F_{(1,32)}$=0.5990, $p$=0.4446). No interaction between time and ethanol was observed ($F_{(2,64)}$=0.5843, $p$=0.5604).

Interestingly, we found a main effect of HDL pre-treatment ($F_{(1,32)}$=4.273, $p$=0.0469), which induced a decrease in the preference for a saccharine solution in both ethanol and control groups, indicative of the amotivational sign of anhedonia. HDL pre-treatment appears to exert a sustained effect on the motivational state of female animals over time (although no interaction between time and HDL administration ($F_{(2,64)}$=2.546, $p$=0.0863) was found).

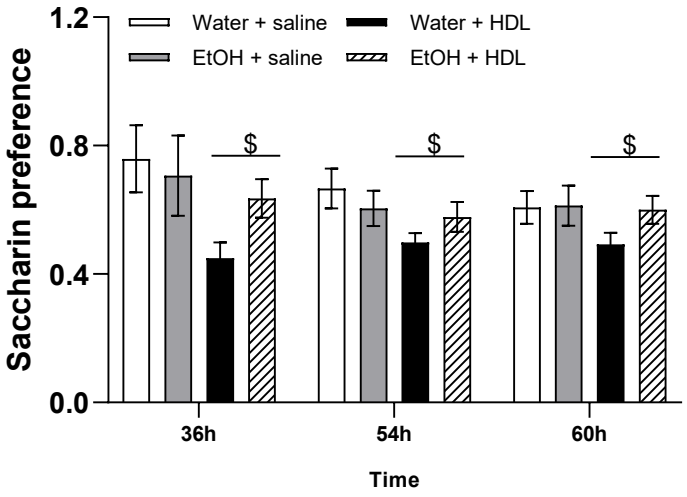



**Figure 33. Saccharine preference test for anhedonia at 36h, 54h, 60h after the last ethanol binge in female animals exposed to alcohol binges and in controls.** The saccharin preference was calculated as the percentage of consumed saccharin solution over the total amount of liquid consumed. A decrease in the preference for a saccharine solution reflects an anhedonic state. Data (n=9) represent the mean ± S.E.M. Repeated-measures 3-way ANOVA: HDL main effect $^{\$}p < 0.05$. *EtOH*=ethanol-treated animals.

## COGNITIVE ASSESSMENT

To study the **spatial memory**, we first performed MWM test. Rodents have been shown to acquire declarative memories when they learn the required route within a specific maze environment (Rendeiro et al., 2009). Thus, as explained in the Method section, 5 days after the last binge administration and during 4 consecutive days, animals were trained four times a day to find the submerged platform in a fixed location of the MWM pool. Latencies to find the platform were recorded in each trial and the average was calculated for each day and animal. The recording of the time spent in the training sessions permits the elaboration of a learning curve for each group and let us know if the animals have learned the location of the platform, in order to perform the probe trial. Animals were considered to have learned if the time taken to find the platform decreased as the sessions progressed.

As shown in Figure 34, control animals performed better on average than the other groups. 2-way ANOVA revealed no main effect of ethanol on any of the training days. However, it was observed that, on average, animals pre-treated with HDL had worse learning during the training sessions, reaching statistical significance on the fourth day (Fig. 34, $F_{(1,32)}$=4.321, $p$=0.0457). Thus, HDL-pre-treated animals took significantly much longer to find the platform during the last training sessions than non-HDL-treated animals.

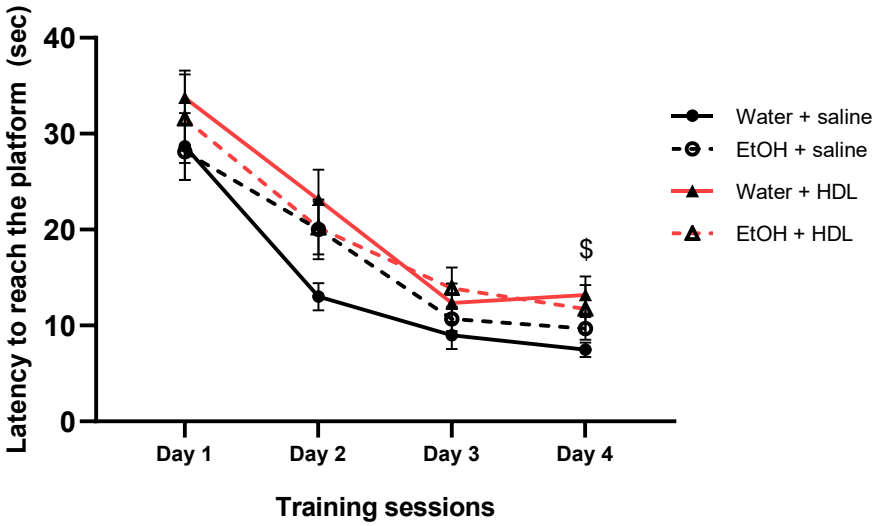



**Figure 34. Learning curve in the Morris Water Maze in female animals in alcohol binge and control groups, pretreated with HDL**. Each day represents the average latency time to find the platform. Animals were considered to have learned if the time taken to find the platform decreased as the sessions progressed. Two-way ANOVA: HDL main effect: $^{\$}p < 0.05$. *EtOH*=ethanol-treated animals.

The fifth day, the test was carried out once without the platform for 60 s with a new start position in the pool to ensure that the rats remember the goal location. Thus, the latency to reach the previous platform location, the number of platform-site crossovers (crosses) and the time spent within an imaginary ring (quadrant) around where the platform had been measured.

2-way ANOVA did not show an ethanol main effect in the latency to reach the platform location or the number of crossings (Fig. 35A,B; $F_{(1,29)}$=0.3059, $p$=0.5844; $F_{(1,32)}$=0.006678, $p$=0.9354, respectively). However, a tendency to decrease was observed in the time spent within quadrant (Fig. 35C; $F_{(1,31)}$=3.279, $p$=0.0799).

In the HDL-pretreated animals, we did not observe HDL main effect in the latency to reach the platform location ($F_{(1,29)}$=0.9646, $p$=0.3342). However, an interaction between factors (alcohol and HDL) was found ($F_{(1,29)}$=10.08, $p$=0.0035). Bonferroni *post hoc* comparisons revealed a significant difference between the control groups (saline vs. HDL), so controls pre-treated with HDL showed higher latency (less memory function) ($p < 0.05$). Although no differences were found in the number of crossings ($F_{(1,32)}$=1.503, $p$=0.2292), an interaction between factors was observed ($F_{(1,32)}$=7.272, $p$=0.0111). Tukey *post hoc* test also revealed differences between control groups (saline vs. HDL, $p < 0.05$). No differences were observed in the time spent in the quadrant ($F_{(1,31)}$=0.00028, $p$=0.9866).

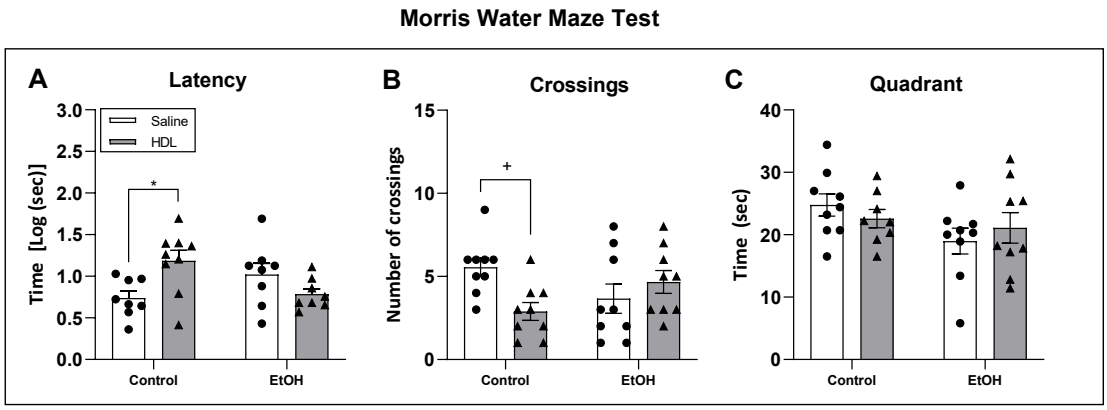



**Figure 35. Spatial memory in the Morris Water Maze in alcohol-intoxicated females and controls, after HDL pre-treatment. A** Latency to find the platform. **B** Number of crossings in the platform location. **C** Quadrant: time swimming in the platform location. Two-way ANOVA: interaction followed by Bonferroni (*) or Tukey (+) *post hoc* test: different from control (water + saline) group $^{*}p < 0.05$, $^{+}p < 0.05$. *EtOH*=ethanol-treated animals.

The **recognition memory** is another type of declarative memory. It is employed to denote the cognitive ability to differentiate between familiar and unfamiliar items, and to subsequently identify these items (Engelmann et al., 2011).

In the short-term, the effects of alcohol abstinence did not induce changes in the recognition memory in females, as evidenced by the DI at 4h after the training phase (Fig. 36A; $F_{(1,32)}$=0.1427, $p$=0.7081). However, HDL pre-treatment caused an increase in the DI ($F_{(1,32)}$=5.017, $p$=0.0322) with no interaction between factors ($F_{(1,32)}$=2.279, $p$=0.1410). In the long-term (24h after the training phase), although no ethanol main effects we observed (Fig. 36B; $F_{(1,31)}$=0.01923, $p$=0.8906), we found again an HDL main effect but in a contrary trend to one described above. Thus, HDL pretreated animals showed lower DI compared to non-HDL treated animals ($F_{(1,31)}$=4.306, $p$=0.0464).

The latency to the first approach to the novel object was also analysed in this test, as a measure of the ability of rats to recognize the novel object before the familiar one. We did not find any effects in the short term 4h after the training phase between groups in this parameter (Fig. 36C). However, in the long-term 24h after the training phase, 2-way ANOVA revealed an ethanol main effect, worsening memory (Fig. 36D; $F_{(1,32)}$=4.978, $p$=0.0328) and an interaction between factors close of significance ($F_{(1,32)}$=3.360, $p$=0.0761). No HDL main effects were observed in the latency in the long-term ($F_{(1,32)}$=1.148, $p$=0.2919).

Thus, in a whole, we observed a worsening effect of alcohol on long-term memory. However, the impact of HDL pretreatment appears to be dual, with a tendency to initially improve the short-term memory in both ethanol and control animals but with a worsening effect in the long-term. In any case, the effects of HDL or alcohol appears to be independent one or each other, since no interactions were found.

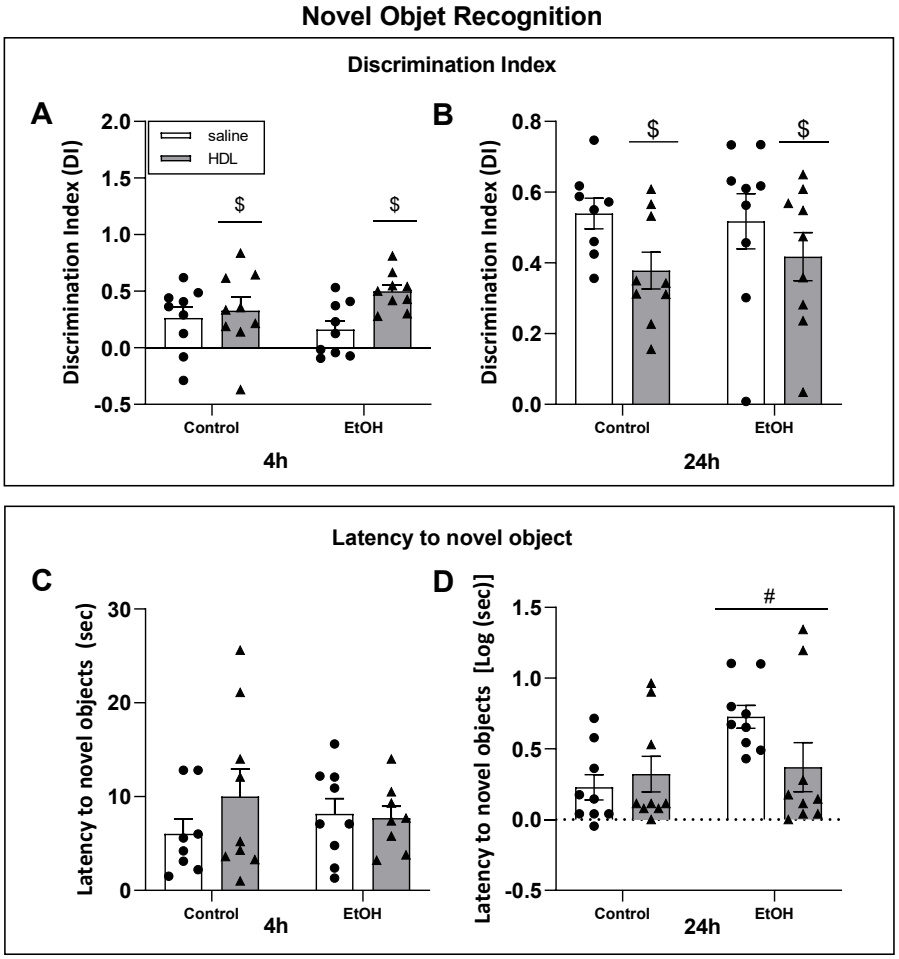



**Figure 36. Novel Object Recognition test to evaluate short- and long-term recognition memory in animals exposed to binge episodes and controls, and the effect of HDL pre-treatment**. **A** Discrimination Index at 4h. **B** Discrimination Index at 24h. **C** Latency to novel object at 4h session. **D** Latency to novel object at 24h session. Results represent the mean ± S.E.M. Two-way ANOVA: EtOH main effect: $^{\#}p < 0.05$; HDL main effect: $^{\$}p < 0.05$. *EtOH*=ethanol-treated animals.

### 7.2.2 Effects of 4F pre-treatment on the neurobehavioural response of females exposed to alcohol binges and controls

Since HDL is composed of different apolipoproteins, being ApoAI its majority apo component (almost 70%), we used a more selective approach to test the effects of ApoAI potentiation in neurobehavioral responses after ABD during abstinence. Thus, we repeated the experiment with females using the ApoAI mimetic peptide 4F, which is a more selective pharmacological tool for this apolipoprotein. Indeed, 4F could be considered more specific as it has been shown to replicate the biological activity of the ApoAI (Gupta et al., 2005; Wolkowicz et al., 2021). In this section, we report the effects of the pretreatment with 4F on behaviour in our ABD model in female animals, following the same experimental design explained in section 6.2.

## EMOTIONAL ASSESSMENT

### Anxiety-like behaviour

12h after the last ethanol binge, ethanol-treated females showed a **disinhibited-like behaviour** by exploring the open arms significantly more than the control groups (Fig. 37A; $F_{(1,30)}$=38.81, $p$<0.0001), as indicated by the percentage of entries. 2-way ANOVA also reported an ethanol main effect in the percentage of time spent in the open arms (Fig. 37B; $F_{(1,33)}$=10.77, $p$=0.0024).

However, no main effects of 4F pre-treatment were observed either in open arms entries or open time ($F_{(1,30)}$=2.279, $p$=0.1416; $F_{(1,33)}$=0.1737, $p$=0.6796, respectively). Moreover, no interaction between factors (ethanol x 4F) were found on any of these parameters ($F_{(1,30)}$=0.06032, $p$=0.8077; $F_{(1,33)}$=0.3997, $p$=0.5316, respectively). It was observed that the disinhibition was an exclusive alcohol effect, and no significant effect was found with the 4F pre-treatment.

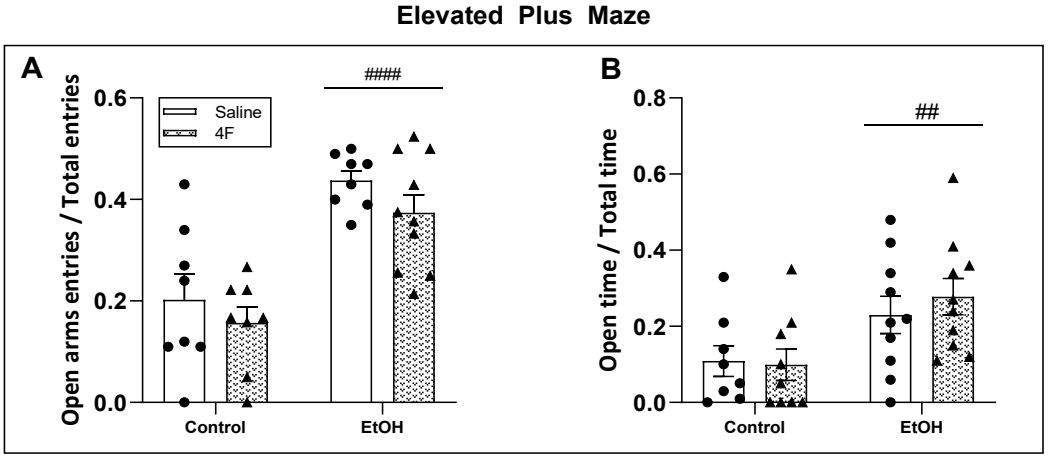



**Figure 37. Effects of 4F pre-treatment on anxiety behaviour in the elevated plus maze in female animals exposed to alcohol binges and controls at 12h of abstinence. A** Number of open arm entries over the total entries into any arm. **B** Time spent in the open arms over the total time spent in both arms. Results represent the mean ± S.E.M. 2-way ANOVA (factors: experimental group x 4F pretreatment): EtOH main effect: $^{\#\#}p < 0.01$; $^{\#\#\#\#}p < 0.0001$. *EtOH*=ethanol-treated animals.

### Depressive-like behaviour and anhedonia

**Depressive-like behaviour** during the abstinence was observed in ethanol-treated female animals in the immobility and latency to first immobility times in the FST (Fig. 38C,D; $F_{(1,33)}$=5.579, $p$=0.0242; $F_{(1,32)}$=5.473, $p$=0.0257, respectively). The cessation of water escape behaviour (as indicated by increased immobility and a decrease in the time spent until the animal relinquishes the attempt, or latency) is regarded as an indicator of negative mood states. No

alcohol main effects were observed in the swimming or climbing times (Fig. 38A,B; $F_{(1,34)}$=0.7681, $p$=0.3869; $F_{(1,34)}$=3.128, $p$=0.0859, respectively).

In 4F-pretreated animals, we did not observe a main effect of 4F on either swimming or climbing time (Fig. 38A,B; $F_{(1,34)}$=1.368, $p$=0.2503; $F_{(1,34)}$=1.388, $p$=0.2469, respectively) nor on immobility or latency to immobility (Fig. 38C,D; $F_{(1,33)}$=2.059, $p$=0.1608; $F_{(1,32)}$=3.265, $p$=0.0802, respectively). No interaction between factors were found in any of these parameters ($F_{(1,34)}$=2.239, $p$=0.1438; $F_{(1,34)}$=0.3819, $p$=0.5407; $F_{(1,33)}$=0.4885, $p$=0.4895; $F_{(1,32)}$=0.02420, $p$=0.8774, respectively).

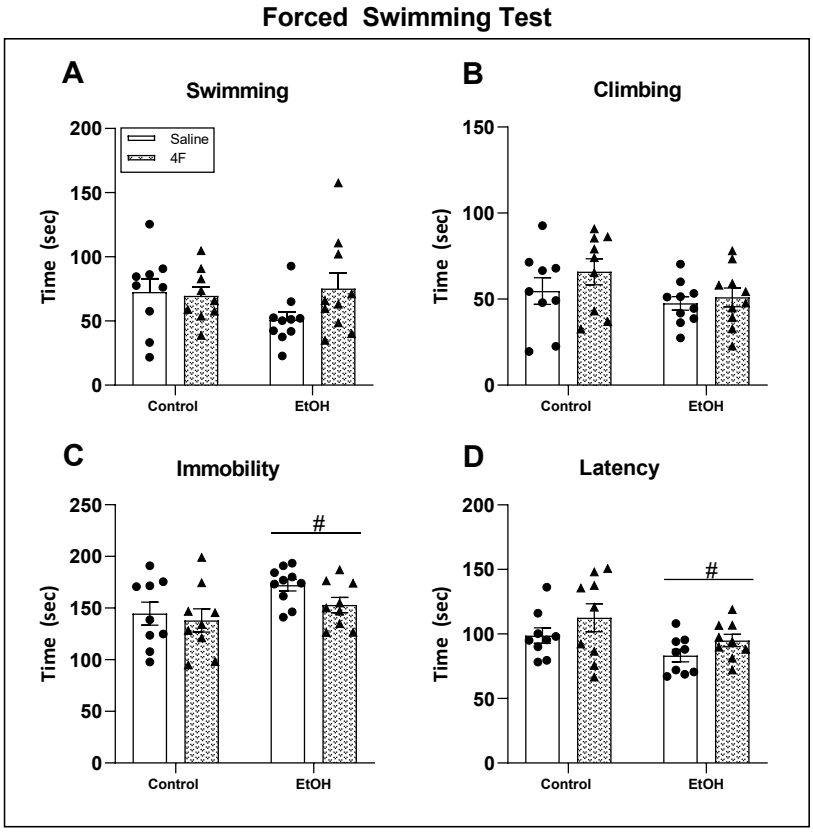



**Figure 38. Forced Swimming test for depressive-like behaviour in female animals exposed to alcohol binges and pretreated with 4F and in control animals. A** Swimming time. **B** Climbing time. **C** Immobility time. **D** Latency to immobility time. Results represent the mean ± S.E.M. Two-way ANOVA: EtOH main effect; $^{\#}p < 0.05$. *EtOH*=ethanol-treated animals.

**Anhedonia**, a core feature of depression, was also measured starting at 36h after the last ethanol binge with the SPT. Figure 39 shows the cumulative saccharin consumption at different measurement times during alcohol abstinence.

Repeated-measures 3-way ANOVA (independent factors: time x EtOH x 4F) found a main effect of ethanol ($F_{(1,34)}$=6.480, $p$=0.0156) with a reduction in the preference for saccharin at all time-points studied, which is indicative of maintained anhedonia during abstinence, an important symptom of depressive-like behaviour. We also observed a main effect of time ($F_{(1.388,42.34)}$=13.44, $p$=0.0002), although no interaction between ethanol and time was observed ($F_{(2,61)}$=1.106, $p$=0.3374).

We did not observe main effects of 4F as it did not modify the preference for saccharine at any time point studied ($F_{(1,34)}$=0.1680, $p$=0.6845). Furthermore, no interaction between 4F and time was observed ($F_{(2,61)}$=0.1758, $p$=0.8392).

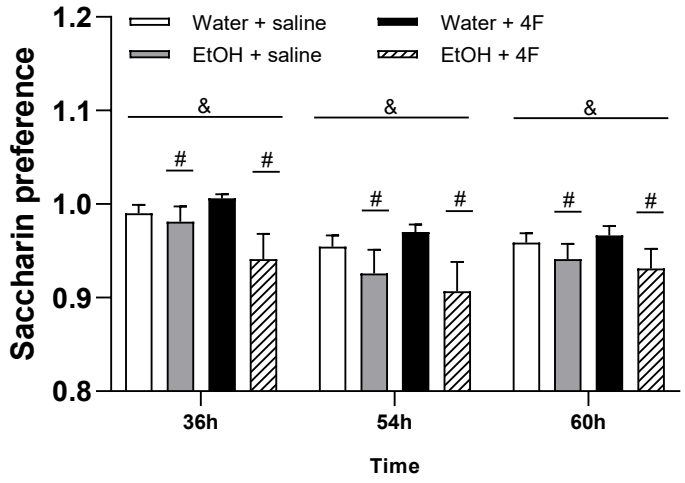



**Figure 39. Saccharin preference test for anhedonia at 36h, 54h, 60h after the last ethanol binge in female animals exposed to alcohol binges and in controls**. The saccharin preference was calculated as the percentage of consumed saccharin over the total amount of liquid. A decrease in the preference for a saccharine solution reflects an anhedonic state. Data (n=9) represent the mean ± S.E.M. Repeated-measures 3-way ANOVA: EtOH main effect $^{\#}p < 0.05$; Time main effect $^{\&}p < 0.05$. *EtOH*=ethanol-treated animals.

## COGNITIVE ASSESSMENT

In the MWM to test the **spatial memory**, and prior to the test day, animals were trained for 4 consecutive days 4 times/day to find the submerged platform. The mean latencies per day are shown in Figure 40. The scores on the first two days are very similar among all groups and 2-way ANOVA revealed no main effect of ethanol on any of the training days. However, we observed a 4F main effect the days 3 and 4 (Fig. 40. $F_{(1,34)}$=4.605, $p$=0.0391; $F_{(1,34)}$=4.225, $p$=0.0476, respectively). 4F-treated animals took significantly much longer to find the platform during the training sessions than non-4F-treated animals.

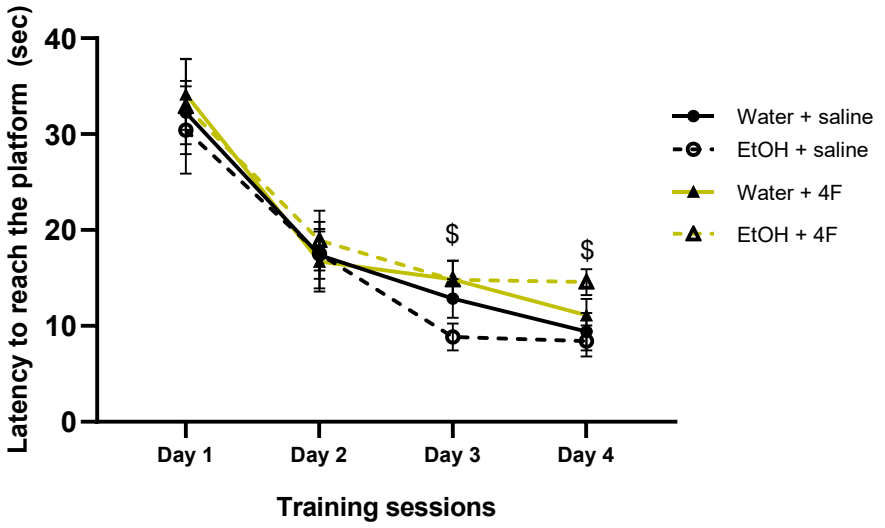



**Figure 40. Learning curve in the Morris Water Maze in female animals in alcohol binge and control groups, pretreated with 4F.** Each day represents the average latency time to find the platform. Animals were considered to have learned if the time taken to find the platform decreased as the sessions progressed. 4F-treated animals had worse learning during the day 3 and 4 as they spent more time to reach the platform. Two-way ANOVA: 4F main effect: $^{\$}p < 0.05$. *EtOH*=ethanol-treated animals.

The fifth day, the test was performed once without the platform for 60 s, using a new start position in the pool to ensure that animals remember the goal location. The latency to catch the previous platform location, the number of crosses and the time in the quadrant were measured.

2-way ANOVA did not show an ethanol main effect in the latency, number of crosses or time in the quadrant (Fig. 41A,B,C; $F_{(1,31)}$=0.1487, $p$=0.7024; $F_{(1,33)}$=0.02798, $p$=0.8682; $F_{(1,34)}$=0.1195, $p$=0.7317, respectively).

In the 4F-treated animals, we found a 4F main effect in the latency (Fig. 41A; $F_{(1,31)}$=4.347, $p$=0.0454) spending more time to reach the platform location. However, no differences were reported in the platform crosses or the time swimming around the quadrant (Fig. 41B,C; $F_{(1,33)}$=0.003109, $p$=0.9559; $F_{(1,34)}$=0.01183, $p$=0.9140). Interestingly, an interaction between factors was found in the latency to reach the previous platform location ($F_{(1,31)}$=6.266, $p$=0.0178) as observed with the HDL pre-treatment (Figure 35A). *Post hoc* comparisons revealed a significant difference between control groups (saline vs 4F) ($p < 0.05$).

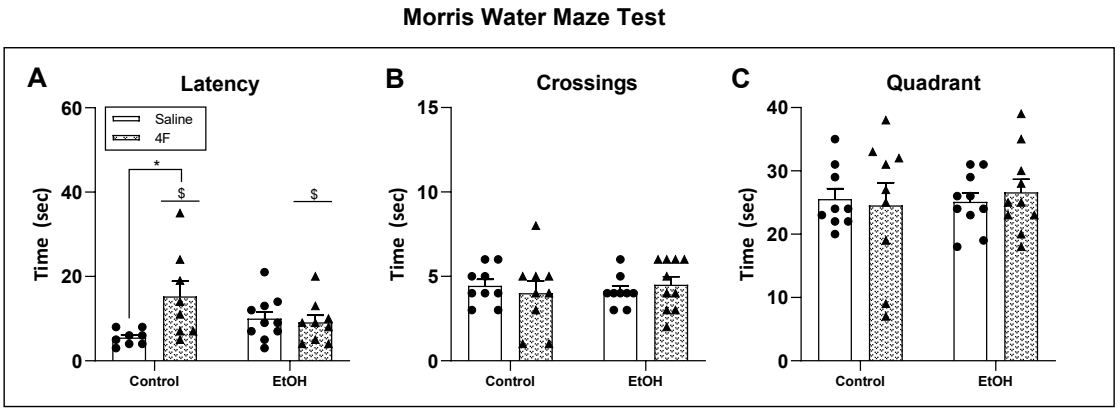



**Figure 41. Spatial memory in the Morris Water Maze in alcohol-intoxicated females and controls, after HDL pre-treatment. A** Latency to find the platform. **B** Number of crossings in the platform location. **C** Quadrant: time swimming in the platform location. Two-way ANOVA: 4F main effect: $^{\$}p < 0.05$; interaction followed by Bonferroni *post hoc* test: $^{*}p < 0.05$. *EtOH*=ethanol-treated animals.

As another measure of declarative memory, NOR test was carried out to study impairment in the **recognition memory**. In the short-term, ABD did not induce changes in the DI at 4h after the training phase (Fig. 42A; $F_{(1,33)}$=2.041, $p$=0.1626), the same effect as observed with the 4F pre-treatment ($F_{(1,33)}$=0.04843, $p$=0.8272), with no interaction between factors ($F_{(1,33)}$=0.3183, $p$=0.5764). However, in the long-term 24h after the training phase, an ethanol main effect was observed, with surprisingly higher DI in the ethanol-treated animals (Fig. 42B; $F_{(1,31)}$=4.221, $p$=0.0484). No effects of 4F pre-treatment or interaction between factors were found in the long-term ($F_{(1,31)}$=1.882, $p$=0.1800; $F_{(1,31)}$=1.319, $p$=0.2596, respectively).

Regarding the latency to the first approach to the novel object, we did not observe an ethanol main effect in the short-term 4h after the training phase (Fig. 42C; $F_{(1,31)}$=1.384, $p$=0.2483). Similarly, no 4F main effects were found ($F_{(1,31)}$=1.201, $p$=0.2816). However, in the long-term 24h after the training phase we found an ethanol main effect (Fig. 42D; $F_{(1,24)}$=4.637, $p$=0.0415), as the ethanol-treated animals displayed lower latency, which is in line with the results of DI described above. No 4F main effects were observed in the latency in the long-term ($F_{(1,24)}$=0.3361, $p$=0.5675).

In this case, we observed main effects of alcohol on long-term memory but contrary to what we observed with the HDL study, where alcohol-treated animals showed higher latencies to novel object (Figure 36D).

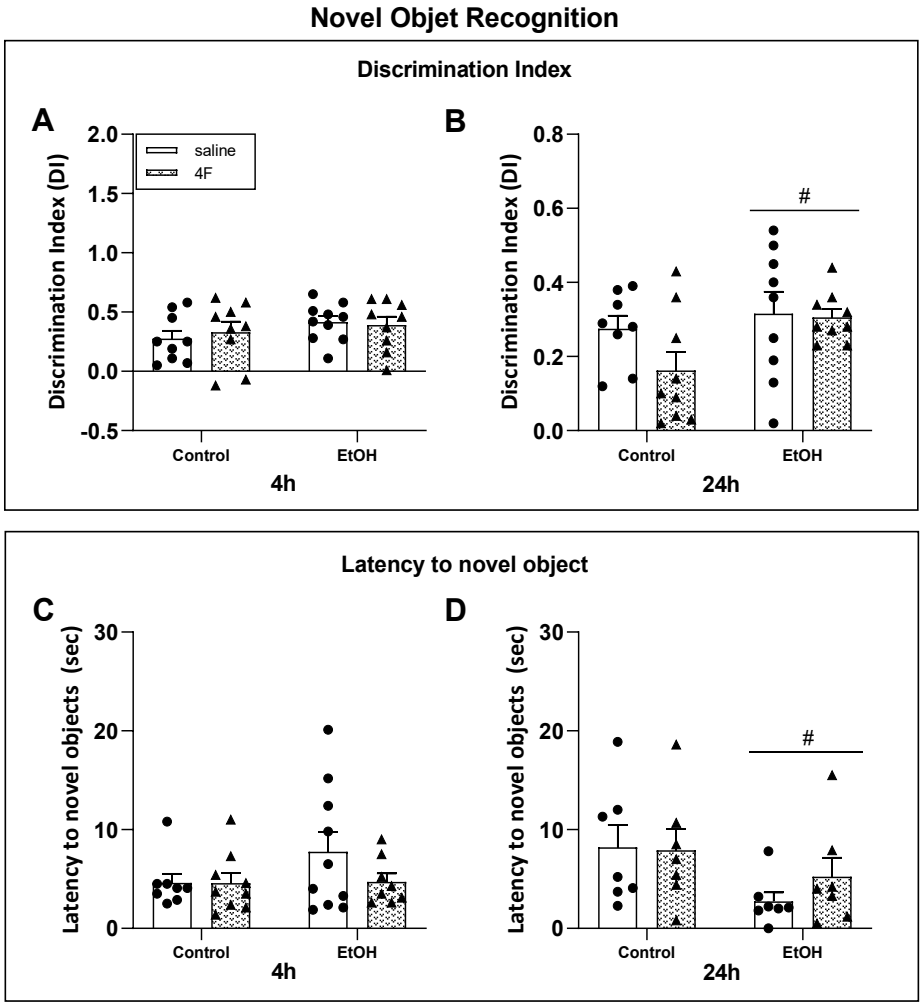
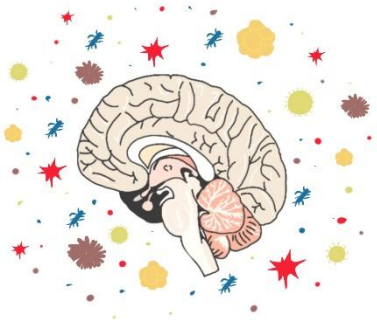



**Figure 42. Novel Object Recognition test to evaluate short- and long-term recognition memory in animals exposed to binge episodes and controls, and the effect of 4F pre-treatment. A** Discrimination Index at 4h. **B** Discrimination Index at 24h. **C** Latency to novel object at 4h session. **D** Latency to novel object at 24h session. Results represent the mean ± S.E.M. Two-way ANOVA: EtOH main effect: $^{\#}p < 0.05$. *EtOH*=ethanol-treated animals.

# 8. DISCUSSION



The studies in this PhD thesis make a valuable contribution to the scientific knowledge on the effects of binge drinking, helping to understand the relationship between alcohol, neuroinflammation and neuroimmune response. We aimed to study the translocation of LPS or its small components to the brain under intensive alcohol consumption and the role of apolipoproteins in this process. Thus, the relationship between ABD and apolipoproteins has been explored for the first time, building on extensive previous research in the context of cardiovascular diseases. Specifically, we studied the involvement of certain apolipoproteins (ApoAI, ApoB and ApoE) in the formation of aggregates with parts of LPS, such as Lipid A, in two brain structures impacted by alcohol abuse (PFC and cerebellum), as well as the expression of apolipoprotein-related receptors. To ascertain the possible functional consequences of these aggregates, we used pre-treatments that mimic the biological action of ApoAI (the apolipoprotein that aggregates with Lipid A in females) and explored their effect in alcohol-induced neuroinflammation and -associated behaviours during abstinence in female animals.

It is important to note that this Doctoral Thesis explores sexual differences in IAC-induced alterations in the expression and binding of apolipoproteins and also behavioural alterations after IAC in females, subjects on which there has been little research until now since most studies on this topic have been conducted only in males. This Thesis also explores for the first time the effects of apolipoprotein mimetic treatments, such as ApoAI-enriched HDL or ApoAI mimetic peptide 4F in the context of alcohol consumption.

Briefly, the main findings of this Doctoral Thesis have shown that ABD induces a translocation of small components of LPS from the periphery to the brain (See figure 43). In the Study I, both Lipid A and core were detectable in brain areas (PFC and cerebellum) in control and ethanol-treated animals, with no significant changes in these free components due to ABD procedure. However, Lipid A, the endotoxic component of LPS, were found bound to specific apolipoproteins within the PFC of animals exposed to alcohol binge intoxications but not within the cerebellum, suggesting a regional difference in the IAC effects regarding this process. In particular, Lipid A was detected bound to ApoAI in the PFC of alcohol-exposed female rats, whereas in male ethanol-treated rats Lipid A was found bound to ApoB, showing a sex-specific interaction between Apos and Lipid A after alcohol exposure. This process might have consequences in alcohol-induced neuroinflammation during abstinence. No alterations in free apolipoproteins within the brain were found either. The presence of LipidA-apolipoprotein aggregated in the PFC of animals exposed to ABD could be related with the well-known disruption of the BBB after this pattern of alcohol consumption in this brain structure (Rodríguez-González et al., 2023; Rubio-Araiz et al., 2017). LipidA-ApoAI binding in ethanol-treated females could be perceived as either a neuroprotective response, as has been observed in other

pathologies (Niu et al., 2024; Z. Zhang et al., 2009), or as a mechanism to shuttle LPS to the brain, as has been suggested elsewhere (Radford-Smith et al., 2023). This topic will be discussed in more detail in this section.

Additionally, alcohol-induced neuroinflammation may have implications in emotional and cognitive alterations during abstinence. In Study II, we explored behavioural changes in females after ABD, highlighting differences compared to the known behaviour of males. This study also examined the potentiation of ApoAI mimetic peptides to modulate the neurobehavioural alterations observed during ABD abstinence, yielding interesting results, contradicting the initial hypothesis in terms of neuroinflammation and behaviour. These findings will be discussed in more detail below.

All these main findings of this Doctoral Thesis will be discussed in this section, subdivided in three differential conceptual blocks.

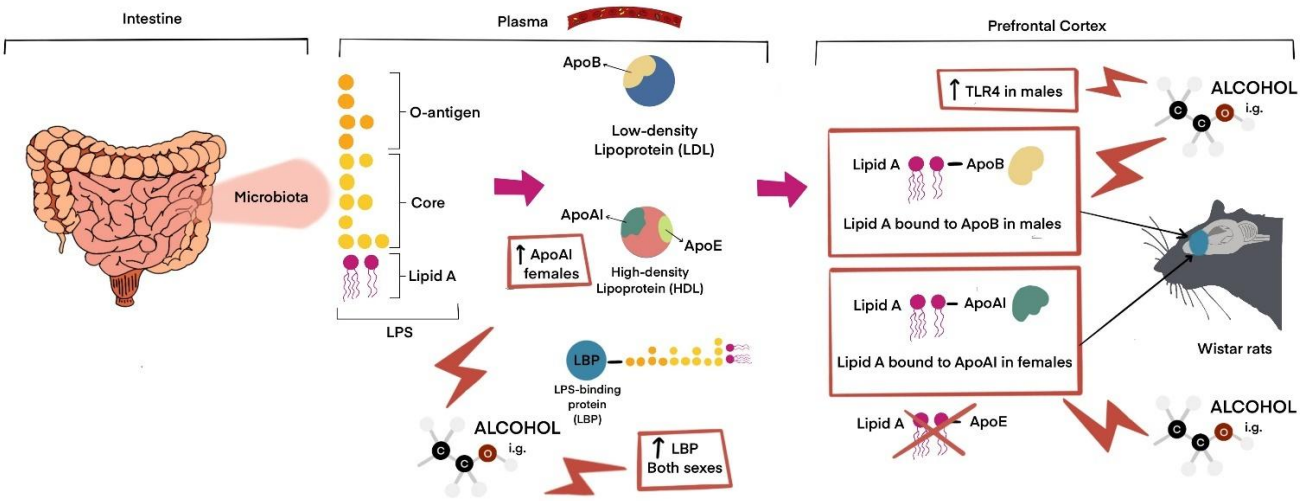



**Figure 43. Graphical abstract of main results of Study I of this Doctoral Thesis** *(own source, generated using Procreate)*

### 1. Effects of alcohol binge drinking model in plasma components

Different **BELs** were achieved with the same doses of alcohol in males and females. Males reached BD levels (>80 mg/dL) whereas lower BELs were observed in females at the time of blood extraction. Indeed, females did not show BELs of BD (<80 mg/dL) at the time of measurement. It is possible that the metabolism of alcohol is faster in females, as some authors have suggested elsewhere (Baraona et al., 2001; Desroches et al., 1995; Kishimoto et al., 2002; Thomasson, 2002), and the peak of BELs occurred at early times before the time-point of blood extraction.

It is well-known that rodents metabolize alcohol faster than humans (in rats, 3 times greater) (Brandon-Warner et al., 2012; Jeanblanc et al., 2019). In this study, we explored the expression of **ADH** and **ALDH**, the hepatic enzymes involved in alcohol metabolism, in order to better understand the differences in BELs in males and females. Some authors have shown sex differences in the elimination of ethanol, being faster in females than males, because the activities of hepatic enzymes appear to be higher in females than male mice (Kishimoto et al., 2002). Rats show a similar pattern as mice, observing higher levels of ADH in females (Elena Quintanilla et al., 2007; Simon et al., 2002), although other authors found no differences (Livy et al., 2003; Lopez et al., 2003). This activity may be associated with a faster ethanol elimination rate from the blood in females than males, showing less BELs than males. In our study, as ethanol-treated females showed less BELs, it was expected that ADH expression would be upregulated. However, we did not observe basal sexual dimorphism in this respect. Unlike this, we found a trend to higher levels of this enzyme in ethanol-treated males vs. control males (near of statistical significance) that is not present in females, which may suggest a faster transition from alcohol to acetaldehyde in males. It is important to note that ADH levels and activity appear to be higher following treatment with ethanol, although we did not find this effect in ethanol-treated females (Eaton et al., 2022).

Regarding **ALDH** levels, a basal sexual dimorphism was found, since control females showed higher liver ALDH levels than control males. Surprisingly, ethanol-treated females showed lower expression of this enzyme, suggesting that females were exposed to acetaldehyde for longer than males after ethanol binges. This would be in line with some authors suggesting that females may be more vulnerable to alcohol toxic effects than males (Alfonso-Loeches et al., 2013; Vandegrift et al., 2017). However, other authors have shown that female rat brains appear to be more resistant to oxidative damage (Jung & Metzger, 2016). Although it did not reach statistical significance, ethanol-treated males showed on average higher levels of ALDH in comparison to male controls, which would suggest a faster ethanol metabolism in males but not females, contrary to what is mentioned in the literature. The study of these hepatic enzymes was

not a main objective in this Thesis, and it represents only a preliminary approach to explain the sex difference in BELs found in our study. Further in-depth study is required to achieve a more comprehensive understanding of the matter.

Some authors reported that differences in alcohol metabolism are believed to be hormonal depending on the **estrous cycle**, but others have found no differences in the metabolic rate of ethanol during the different stages of the estrous cycle (Brick et al., 1986). In our study, the phases of the estrous cycle do not seem to alter BELs, as we found no correlations between them (data not shown). Biological differences between males and females and hormonal factors could interfere with the responses observed, but comparative studies showing the influence of ethanol in males and females using the same experimental approach at a time are still scarce. For example, sex hormones in females may contribute to a possible neuroprotector effect, as has been suggested elsewhere (Jung et al., 2005; Zárate et al., 2017). In this study, the reproductive cycle was monitored in the females during experimentation, and they exhibited regular 4 to 5-day estrous cycles throughout the experimental testing period. No synchronization among them and no variation in BELs values across estrous cycle phases were observed.

Despite the lower BELs at the time of blood measurement, females in the ethanol group showed the activation of the acute-phase response, as indicated by the raise in plasma **corticosterone** levels. This higher corticosterone response in females than in males after alcohol exposure is consistent with the existing literature (Marco et al., 2017; Nentwig et al., 2019; Rivier, 1993).

It is well known that alcohol intoxications induce an increase in **plasma LPS** due to leaky gut and liver clearance downregulation (Antón et al., 2017, 2018; Leclercq et al., 2012; Orio et al., 2018; Schaffert et al., 2009; Stärkel et al., 2018). In this study, while there was an overall effect of alcohol, the increase in LPS did not reach statistical significance (near of statistical significance). This was surprising, since previous studies to this Thesis in our laboratory had reported LPS increases under the same binge procedure in males (Antón et al., 2017, 2018; Rodríguez-González et al., 2021), and it is probably attributed to the slightly younger age of these animals compared with the mentioned studies (Walker & Ehlers, 2009). Nevertheless, we observed a clear significant increase in LBP in both females and males in response to ABD, which has also been considered a marker of bacterial translocation and inflammation (Kumar et al., 2024; Stehle et al., 2012).

**ApoAI**, the main Apo constituent of **plasmatic HDL**, is predominantly synthesised in the liver and intestine (Meilhac et al., 2020) and, as mentioned before, it is responsible for the removal

of excess cholesterol from cells and the transport of LPS to the liver for its elimination (Berbée et al., 2005; Corraliza-Gomez et al., 2019). In this study, no basal dimorphisms were found in the concentration of ApoAI in plasma between male and female control animals, although it has been reported that women exhibit higher plasma HDL-ApoAI levels under physiological conditions compared to men (Conlon et al., 2023; X. Wang et al., 2011). However, we found an increase in plasma ApoAI levels in the female ethanol group but not in males, despite no changes in plasma HDL were found. These findings are consistent with recent studies suggesting that, under pathological conditions, HDL functionality is more influenced by the HDL protein cargo than by changes in HDL plasma levels. (i.e., ApoAI) (Davidson et al., 2022; Pedrini et al., 2022). In humans, moderate alcohol consumption has been associated with increases in plasma HDL levels (Hannuksela et al., 2004) in a dose-dependent fashion and turnover of ApoAI (De Oliveira E Silva et al., 2000), although recent studies suggest that long-term alcohol consumption may decrease HDL serum levels in women (Cho et al., 2022). Several studies have shown that an increase in LPS within the circulation during an infection can induce dyslipidemia. In the context of dyslipidemia, a decrease in HDL and alterations in lipoprotein composition (i.e., a reduction in ApoAI expression in HDL) have been observed. These changes have been associated with an elevated risk of mortality due to sepsis (Tanaka et al., 2020). It is known that, under inflammatory conditions, ApoAI can be replaced by serum amyloid A (SAA), reducing the ApoAI content and depleting the ability of HDL in the liver to neutralise endotoxin-mediated inflammation (Meilhac et al., 2020; Shah et al., 2013; Tanaka et al., 2020). In our study, we observed increases in free ApoAI plasma levels in females that underwent ethanol intoxications. However, we are unaware of the subsequent levels of ApoAI within HDL or whether it was replaced by SAA, which may be a limitation of our study.

**Plasma LDL**, which transports mainly **ApoB**, plays an important role in lipid transport (Anita C.E. et al., 2001; Corraliza-Gomez et al., 2019; K. Singh & Prabhakaran, 2024), and it appears to be higher in men than in women (X. Wang et al., 2011). In agreement, peripheral dimorphisms were also identified in physiological conditions in our study, with males demonstrating higher plasma LDL and ApoB levels in comparison to females, regardless of the influence of alcohol.

It is well established that alterations in the metabolism of plasma Apos and lipoproteins have been identified in cases of brain disorders, with schizophrenia being a particular example (Boiko et al., 2019; J. T. J. Huang et al., 2008). Further alterations resulting from alcohol abuse have been described in the context of metabolic syndrome or alcohol-induced alterations in energy homeostasis, obesity and liver diseases (K. Sun et al., 2014). Reports about the effect of intensive alcohol consumption on plasma lipoproteins are very scarce in the field, with some reporting

increases in plasma HDL and decreases in LDL profiles in heavy drinkers (Wakabayashi, 2013). These results highlight one of the sex differences found in our study: ApoAI plasma levels were increased in females under ethanol intoxications and this effect was not found in males. The higher levels of plasma ApoAI found in female animals of the ethanol group could indicate that females activate an ApoAI-mediated protective mechanism to neutralize LPS, since it is known that HDL, which integrates mainly ApoAI in the periphery, binds LPS and helps in its transport to the liver for elimination (Berbée et al., 2005; Brandenburg et al., 2002). The reason why this increase happens only in females but not in males, having both a similar plasma LPS/LBP activation after ABD, remains unknown and it will be discussed in base of the results found in the brain (see section 7.1.5).

### 2. Detection of LPS components (Lipid A and core) and apolipoproteins in the brain of male and female animals after ABD: aggregates formation

We explored the presence of small components of the LPS molecule, such as Lipid A and Core, in brain structures. Lipid A and Core were detected in the PFC and cerebellum of both sexes, but there were no significant differences between the control and ethanol groups in any of these structures. It is important to note that these brain structures are well-protected by the BBB, although minimal penetration of labelled LPS with radioactive iodine (I-LPS) across the BBB has been studied before (Banks & Robinson, 2010).

**ApoAI** was detected in the PFC and cerebellum of both male and female rats, but no differences on the expression of the free form (non-bound) were identified after ethanol binges in any of these structures. As previously described in the introduction, ApoAI is also present in the brain naturally, as it has been demonstrated to cross the BBB back and forth between the circulation and the brain (Balazs et al., 2004; Cho, 2022) via SR-BI, the main lipoprotein receptor for ApoAI expressed in endothelial (Balazs et al., 2004) and glial cells (Hottman et al., 2014), responsible for mediating ApoAI endocytosis in the CNS. ApoAI has also been identified in the CSF (Camacho et al., 2019; Ito & Michikawa, 2014; Lefterov et al., 2010). Here, we did not find differences in the SR-BI expression in the PFC or cerebellum in either females or males after IAC. Interestingly, it was observed that Lipid A was specifically bound to ApoAI in the PFC of female rats exposed to alcohol, but not in the controls. This effect was not observed in male animals (instead, a binding to ApoB was identified), showing a sex-specific interaction between Apos and Lipid A. This finding is open to a number of interpretations, as will be discussed below.

As with ApoAI, **ApoB** was detected in the PFC and cerebellum in both sexes with no differences on its expression in either males or females after ABD. However, unlike ApoAI, it is believed that ApoB cannot cross the BBB (Cho, 2022), and it is mainly synthesized in the liver. Nevertheless, some studies have corroborated the presence of ApoB in brain endothelial cells in mice (Lénárt et al., 2015), suggesting that ApoB could cross the BBB indeed. As mentioned before, some studies found brain ApoB bound to αβ plaques in transgenic AD mice (Martins et al., 2009; Takechi et al., 2009), crossing from blood to the brain via LDLr, which are expressed in neurons and glial cells (Corraliza-Gomez et al., 2019), by transcytosis (Dehouck et al., 1997). Here, we detected an upregulation of LDLr expression in the PFC (but not in cerebellum) of male animals that underwent alcohol administrations.

The presence of LipidA-Apo aggregates was only found in the PFC but no in the cerebellum. As previously mentioned, ABD has the capacity to modify TJs expression in the BBB in the PFC, thereby impacting the integrity of this barrier and potentially facilitating the passage and/or signalling of peripheral components. However, there is currently a lack of knowledge regarding ABD and the BBB in the cerebellum, with only a limited number of studies reporting alterations in the BBB in the cerebellum due to inflammation induced by viruses or bacteria (Muller et al., 2005; Phares et al., 2006).

We also checked the presence of **ApoE**, one of the major apolipoproteins in the CNS. which is highly expressed by astrocytes, oligodendrocytes and microglia (Mahley, 2016; J. Zhang & Liu, 2015) and involved in the transport of cholesterol and other lipids through the bloodstream and the CNS (Camacho et al., 2019; Lefterov et al., 2010), together with the ApoAI. The present study revealed no differences in the expression of ApoE between the experimental groups or its binding to Lipid A in the PFC and cerebellum, both in males and females. It should be noted that we did not differentiate between ApoE isoforms, which is probably a limitation of the study. The ApoE4 isoform has been linked to neuropsychiatric diseases and extensive research associate it with neuroinflammation and cognitive decline (Aboud et al., 2014; Duro et al., 2022; Montagne et al., 2020). ApoE4 is a well-known risk of AD (Golden & Johnson, 2022; Jackson et al., 2024; Shi et al., 2014) and it has been studied in the context of alcohol abuse (Downer et al., 2014; Escudero et al., 2023).

The levels of ApoE in plasma were under the limit of detection in our experiment. It is believed that brain ApoE does not cross to the periphery, but it may cross the BBB when it is bound to HDL (Pedrini et al., 2022). Indeed, the primary source of peripheral ApoE appears to be mainly the liver, with minimal contributions from the brain and endocrine cells to plasma ApoE levels (Pedrini et al., 2022). In our experiment, we did not study the levels of HDL within the

brain, as HDL in the periphery is mainly enriched with ApoAI, and its synthesis in glial cells is enriched with ApoE (Pedrini et al., 2022; Van Valkenburgh et al., 2021). In any case, ApoE appears not to play a fundamental role in the hypothesis of this study, although given the importance that the isoform ApoE4 plays in the context of alcohol and neuroinflammation, further studies are needed to ascertain possible implications of this specific isoform of ApoE.

There are several potential explanations for the binding of Lipid A-ApoAI in the PFC. Firstly, elevated levels of peripheral ApoAI in female ethanol group could bind Lipid A in the plasma, with the complex then being transported to the blood-brain interfaces or brain parenchyma within the PFC; secondly, the LipidA-Apo aggregate could occur within the BBB structures (i. e. endothelial cells) in the PFC at the luminal (blood-facing) or abluminal (brain-facing) side of it; third, both possibilities could be happening at the same time. In any case, these findings would suggest that LPS components may signal in the PFC, but not in the cerebellum, following alcohol binge intoxication, potentially facilitated by an apolipoprotein-dependent mechanism. These hypotheses need further confirmation in future studies, as well as the precise mechanisms and the functional consequences of the binding. Moreover, SR-BI appears to be responsible for HDL internalization and transcytosis across the BBB (Fung et al., 2017), but its participation in the processes described here is uncertain, since no changes in the expression of SR-BI were observed in any condition in this study.

To ascertain the influence of blood flow within the brain in the changes previously discussed, we performed a pilot study comparing perfused (in which blood has been removed) and non-perfused female control rats. This pilot study demonstrated that in perfused control animals (in which blood has been removed) the free Lipid A has lower expression than in intact (non-perfused) brains. This result suggests that a percentage of Lipid A observed in this study is located outside of the BBB, and that another percentage may infiltrate it. This finding is consistent with mechanistic studies that have shown that, after perfusion with I-LPS, approximately half of the I-LPS associated with then brain permeated the BBB, entering the parenchyma space, while the remaining 50% was sequestered by the capillary bed into endothelial cells (Banks & Robinson, 2010).

The location of Lipid A on the luminal (blood-facing) or abluminal (brain-facing) side of brain endothelial cells was not investigated in this study, since it was not an objective of the present Thesis. However, it is known that LPS may bind to receptors located in brain endothelial cells, including TLR4, inducing the release of proinflammatory cytokines, and this release can occur on either side of the brain endothelial cells (Verma et al., 2006). Interestingly, it was observed that the aggregates were maintained in perfused animals at levels that were similar to

those in non-perfused rats. Thus, the clearance of the vascular space did not alter the expression of LipidA-Apo aggregates within the brain in our pilot study, suggesting that they may infiltrate the brain. This was also reported by some authors in a previous immunohistochemical study, where the presence of analogous aggregates in the blood-brain interfaces was identified, including tanycyte-like cells, ependymal cells, and brain endothelial cells, as well as within the cerebral parenchyma, such as astrocytes in the medulla oblongata (Vargas-Caraveo et al., 2017). It is conceivable that the Lipid A-Apo aggregates are components of the BBB (i.e. endothelial cells) and play a role in maintaining vascular homeostasis, impacting proinflammatory signals on either side of the barrier (A. K. Singh & Jiang, 2004; Verma et al., 2006).

**Heads or tails?: Is the LipidA-ApoAI binding a transport system or is a protective mechanism in the brain?**

As explained in the Introduction, HDL, and its main Apo, ApoAI, contribute to LPS detoxification in the liver in physiological conditions, functioning as vital elements of the innate immune system (Berbée et al., 2005; Phillips, 2013). Here, we detected the presence of Lipid A, the endotoxic part of LPS, bound to ApoAI in the PFC of ethanol-treated females. The presence of the LipidA-ApoAI aggregates coincides with the lack of the overexpression of neuroinflammatory markers in the PFC. Thus, TLR4 (sign of neuroinflammation) and TNF-α or IL-1β (note a tendency here) were not upregulated in females exposed to ethanol. In this way, the sequestration of Lipid A by ApoAI in the female brains after IAC could be interpreted as a mechanism to protect it against neuroinflammation, preventing a LipidA-mediated activation of TLR4 signalling, although we cannot discard other explanations. This potential protective effect did not appear to occur with the ApoB aggregates in males, where TLR4 was clearly upregulated in the PFC after ABD, as observed in previous studies (Alfonso-Loeches et al., 2010; Antón et al., 2017; Orio et al., 2019). The anti-inflammatory actions of ApoAI have been described in several conditions (Tao et al., 2024), such as sepsis (Z. Zhang et al., 2009). Decreases in plasma ApoAI have been associated with the severity of Alzheimer´s disease (AD) (Niu et al., 2024; Pedrini et al., 2022), or even decreases in brain and plasma ApoAI have been related to schizophrenia (Boiko et al., 2019; J. T. J. Huang et al., 2008). Clearly, further research is needed to ascertain a possible protective role of ApoAI in the female brain under alcohol conditions. Intriguingly, in a very recent study published during the course of this Doctoral Thesis, Radford-Smith and colleagues examined the effect of intraperitoneal administration of HDL together with LPS in mice. Their findings suggest that HDL shuttles the endotoxin LPS to the brain, thereby inducing neuroinflammation. In contrast, the co-administration of LDL with LPS had anti-neuroinflammatory properties (Radford-Smith et al., 2023). Then, the binding of Lipid A with ApoAI could be interpreted as a protective strategy to capture LPS, in line with most of the

studies, or, counterintuitively, as a strategy to help LPS access the brain, as suggested the mentioned study of (Radford-Smith et al., 2023).

In order to check the possible anti- or proinflammatory actions of ApoAI we performed the Study II, in which we administered HDL or ApoAI mimetic peptide exogenously to study neuroinflammatory and behavioural parameters after ABD. Briefly, although our Study I suggested a possible anti-inflammatory effect of the ApoAI aggregates, the exogenous potentiation of this apolipoprotein did not have anti-inflammatory effects or ameliorate neurobehavioral responses after intensive alcohol consumption. These results will be extensively discussed below.

Anyhow, it has been suggested that lipoproteins may have dual functions. Firstly, they may capture and clear LPS from the blood and tissues. Secondly, they may induce inflammatory responses elsewhere (Radford-Smith et al., 2023). So, it is possible that the effects of the ApoAI depends on several factors, including the time course of the neuroinflammation or its incorporation to different lipoproteins, and further research is needed to ascertain this point. Indeed, it has been established that between 80–97% of LPS is bound to lipoproteins in the bloodstream, with all main lipoprotein classes being involved in a complex way (Kallio et al., 2008). As mentioned in the Introduction, the first step in LPS detoxification is binding to HDL, being reduced its inflammatory activity. However, the 'buffering' capacity of HDL is quickly exceeded, and LPS should be moved to LDL (Walley, 2016). Thus, while LPS demonstrates a higher affinity for HDL, it can be transferred from HDL (ApoAI) to LDL (ApoB) in response to an acute-phase response to infection (Levels et al., 2005). However, the capacity of lipoproteins to detoxify LPS may also be subject to negative regulation in inflammatory scenarios, where certain oxidative stress mediators could oxidise Apos, resulting in the production of dysfunctional lipoproteins (oxHDL, oxLDL) (Meilhac et al., 2020). Further research is required to establish whether the binding of LPS to different lipoproteins/apolipoproteins is effective in buffering the pro-inflammatory potential of the endotoxin. In this regard, some authors have suggested that the shift of LPS towards lipoproteins with lower densities may result in less effective endotoxin scavenging (Kallio et al., 2008). This finding aligns with the observations made in our study, where we found that in alcohol-treated animals, Lipid A is bound to ApoAI (HDL) in the PFC of females which coincides with a lack of neuroinflammation, while it is bound to ApoB (LDL) in males, who exhibited a more pronounced neuroinflammatory response in the PFC. It is possible that differences in the magnitude of the acute-phase response induced by alcohol in males and females in our study account for these differences, since LPS may be exchanged between lipoprotein subclasses according to the neuroimmune capacity, as discussed above.

Notably, one of the molecules responsible for this transfer of LPS from HDL to LDL is plasma LBP, participating in a second step in the LPS detoxification process (Levels et al., 2005). Some authors have suggested that LDL is a predominant LPS binding lipoprotein by means of a mechanism related to increased levels of LBP as cofactor (Vreugdenhil et al., 2001). This is also in line with our results, as we found increased levels of plasma LBP in all ethanol-treated animals versus controls, together with the higher plasma LDL and ApoB profile only in our male animals. LBP is considered an even stronger neutralizing molecule against LPS-induced inflammation than ApoAI (Wurfel & Wright, 1995), suggesting that the female ethanol-treated rats in this study were double protected by elevations in both ApoAI and LBP plasma levels, whereas ethanol-treated males could have a less effective endotoxin scavenging with higher LDL/ApoB plasma levels.

### 3. The effects of ApoAI potentiation by exogenous administration

As explained before, we found LipidA-ApoAI aggregates in the brain of female animals exposed to alcohol intoxications but not in males, which could initially be interpreted as a homeostatic response to counteract alcohol-induced neuroinflammation, since females showed reduced inflammatory levels than males. The literature also mainly indicates anti-inflammatory properties of ApoAI (Fotakis et al., 2019; Gupta et al., 2005; Sviridov et al., 2008; Tao et al., 2024), as exposed previously. However, emerging evidence indicates a complex effect of lipoproteins in the inflammatory processes, and even a proinflammatory role of HDL in the brain after exogenous administration (Radford-Smith et al., 2023).

So, we aimed to explore the effect of an exogenous administration of HDL or an ApoAI mimetic peptide. Additionally, since male and female showed different brain aggregates to be studied, we prioritized the study with females, due to the scarcity of research on females in comparison to males in this field.

ApoAI, the main Apo in plasma HDL, has recognised protective properties in cardiovascular diseases (Fotakis et al., 2019; Sviridov et al., 2008) and other fields of biomedicine, such as virology (Coelho et al., 2021), using mimetic peptides like 4F. In the context of endotoxemia, circulating lipoproteins play a vital role in defending the host. Several studies have shown that HDL levels are found decreased in case of sepsis, while increasing HDL concentration has the potential to reduce complications associated with endotoxemia in both rodents and humans (Reisinger et al., 2022; Tanaka et al., 2020; A. Wu et al., 2004). Thus, elevating lipoprotein levels could offer a promising therapeutic approach to mitigate or neutralise the toxic effects of LPS (Khovidhunkit et al., 2004). However, whether these protective effects

could have any impact on the known neuroinflammation caused by alcohol and its behavioural consequences have not been studied.

The intraperitoneal administration of exogenous HDL 15 min before each ethanol binge promoted higher levels of LPS in plasma both in control and experimental conditions, independently of the effect of alcohol. This effect was not observed in the brain, although animals that underwent ethanol binges showed higher levels of brain LPS on average (a trend, non-significant effect). HDL may increase plasma LPS under both physiological conditions and with alcohol in females. This effect in control animals was unexpected and remains to be studied. Interestingly, only ethanol-treated animals showed elevated plasma ApoAI, which may be a key factor to conform the LipidA-ApoAI aggregates found within the brain. So, it is likely that only small parts of LPS, such as Lipid A, cross into the brain only when aggregated with ApoAI. Essentially, IAC does not necessarily lead to higher levels of Lipid A or LPS in the brain, but rather to an increased presence of aggregates. The formation of LipidA-ApoAI aggregates may be the result of the combined effects of elevated ApoAI (increased in plasma only with alcohol) and higher LPS levels in HDL-associated groups.

ABD has been shown to stimulate the synthesis of pro-inflammatory cytokines, such as TNF-α and IL-1β, both in plasma and in brain tissue (PFC) of male rats following activation of the TLR4 cascade (Antón et al., 2017). In ethanol-treated females, we did not observed overexpression of TLR4 protein or some cytokines, such as TNF-α and IL-1β, after ABD in the PFC in our Study I. In this line, in Study II we only observed an ethanol main effect in the CXCL1 gene expression, involved in the acute phase response. However, the presence of TLR4 gene expression and/or other pro-inflammatory cytokines was not detected in the PFC after IAC at the time of testing. As discussed in other sections, females may metabolize the alcohol differently than males, so the peak of inflammatory markers may be reached at a different time point than males. The inflammatory response is very complex, with a great deal of markers that up- and down-regulate in short periods of time. Anyhow, we found an upregulation of pro-inflammatory gene expression in the PFC after HDL pre-treatment, including TLR4, HMGB1 and CXCL1, as well as a tendency to increase in TNF-α, in both control and ethanol-treated animals, which is in line with the upregulation of plasma LPS levels. Taken together, these results would suggest that exogenous HDL administration promotes a neuroinflammatory environment both in the periphery and the brain and that HDL pretreatment not only did not ameliorate alcohol-induced neuroinflammation but also that endogenous dysregulation of HDL levels could have undesirable consequences in control conditions.

It is important to note that, due to time constraints, we have not been able to study the effects of the 4F pre-treatment on neuroinflammatory parameters (we did only behavioural assessment), although some studies have reported that 4F decreased pro-inflammatory cytokines levels after LPS treatment in human umbilical vein endothelial cell culture (Gupta et al., 2005). Some authors have proposed that the mechanisms by which 4F protects against the effects of LPS are through its direct binding and neutralisation, either by inducing an increase in HDL levels or by facilitating its uptake by HDL particles (Dai et al., 2010). However, the effects of 4F on neuroinflammatory markers in our experimental conditions, during alcohol intoxications, remains to be elucidated in future investigations.

Only a few studies have investigated the effects of HDL on inflammatory cells under other conditions. Some authors suggest that HDL may have pro-inflammatory activity, and it may be implicated in the maintenance of an efficient innate immune responses by macrophages against bacterial infections, such as P. aeruginosa, which is a *Gram*- bacteria (van der Vorst et al., 2017). Thus, in murine models and humans, low levels of HDL and/or ApoAI have been demonstrated to result in delayed and defective innate immune responses, with an exaggerated response after endotoxin administration (Birjmohun et al., 2007; Feingold & Grunfeld, 2016; Guo et al., 2013). However, in the present study, no differences in plasma HDL levels were observed after ABD treatment, although ethanol-treated females exhibited increased plasma ApoAI levels and binding of this protein to Lipid A in the PFC. Thus, it is possible than an endogenous homeostatic mechanism, highly regulated, could be inducing the LipidA-ApoAI aggregates in an attempt to mitigate alcohol-induced neuroinflammation, but exogenous potentiation of ApoAI may facilitate the transport of LPS to the brain, as suggested by the proinflammatory profile found in controls of our study after HDL treatment, and as suggested elsewhere (Radford-Smith et al., 2023; Vargas-Caraveo et al., 2017).

Other authors have shown that ApoAI could activate MyD88, the intracellular adaptor of TLR4, in macrophages, promoting the expression of cytokines and inflammation (Smoak et al., 2010), which would be more in agreement with our data. As mentioned before, exogenous administration of HDL with LPS in mice resulted in the detection of LPS in the brain, suggesting that HDL may act as a transport mechanism for LPS into the brain (Radford-Smith et al., 2023). Interestingly, these animals also showed an upregulation in the gene expression of some pro-inflammatory markers, such as TNF-α and IL-1β, which is in line with our results.

It is important to note that plasma HDL is highly heterogeneous in nature, not only because it is composed of various apolipoproteins (ApoAI, ApoE, etc) but also due to the presence of numerous associated proteins and their implication in several metabolic pathways, being

involved in human metabolism more than is currently appreciated (Davidson et al., 2022; Khovidhunkit et al., 2004). So, we are aware that the administration of the whole HDL (from human origin) is a very unspecific pharmacological tool that helps us only to preliminary test the direction changes of the inflammatory response in our model Some proteomic studies have revealed the presence of hundreds of HDL-related proteins, which appears to be related to inflammation and the immune response, thereby affecting to the HDL functionality (Conlon et al., 2023; Ronsein & Vaisar, 2019; Shah et al., 2013). In addition, inflammation has been shown to cause significant metabolic and structural changes in HDL in mice and humans (Feingold & Grunfeld, 2016; Khovidhunkit et al., 2004; Ronsein & Vaisar, 2017). In our study, the inflammatory response after ABD in females was not very apparent so it is probably not enough to alter the HDL conformation. Indeed, the HDL-induced inflammatory effects appeared both in control and ethanol-treated rats.

The pro-inflammatory effects of HDL pre-treatment affected both HDL groups, including control animals where there is not an inflammatory environment. We did not observe main differences after HDL pre-treatment between experimental groups, as expected, but it is to note that, in females in our study, the neuroinflammatory profile between control and ethanol-treated animals at the time of sample collection was similar, so HDL may have a similar damaging effect in both groups in this case. Our results point out to a negative effect of enhancing ApoAI both in control and ethanol conditions, that differ for the more recognized anti-inflammatory role of the Apo, as mentioned before (Chernick et al., 2020; Dai et al., 2010; Fotakis et al., 2019; Geeta Datta, 2011; Phares et al., 2006).

### 3.1 Neurobehavioural effects of ApoAI potentiation by exogenous administration of mimetic compounds

There is limited knowledge about the behavioural implications of potentiating ApoAI levels during ABD, which is discussed below. It is important to note that we have studied behavioural consequences after IAC in female animals for the first time in our laboratory and this has resulted in difficulties in the interpretation of the results obtained as the literature is very scarce in these subjects, with no female studies following the ABD protocol used here.

IAC either had no effect on **anxiety-like behaviour** during abstinence or had effects contrary to those expected from previous studies, where ABD resulted in a predominantly anxiety-like behaviour in male animals in the EPM during abstinence (Antón et al., 2017; Evans et al., 2020; Gilpin et al., 2012). Whereas EPM testing in males exposed to ABD typically shows an anxiogenic phenotype during abstinence, IAC induced a opposite effect here in females exposed to the binges, independently of HDL or 4F administration. Alcohol-treated females

exhibited an apparent decrease in anxiety-like behaviour, as an increase in the percentage of entries and time spent in the open arms compared to non-alcohol-treated animals were observed. Although the EPM is widely regarded as a model for anxiety-like behaviour, increasing evidence supports alternative interpretations (Davis et al., 2009; Joshi et al., 2018; Molander et al., 2022; Moya et al., 2022). Thus, more entries or more time spent in the open arms in the EPM versus control animals can therefore serve as an index of cortical disinhibition and impulsivity, dependent on FC alterations (Moya et al., 2022).

The responses of ethanol-treated females in the EPM may indicate impaired impulse control and an increased drive towards risky behaviour due to a lack of environmental awareness. Notably, most studies highlighting the anxiogenic role of ABD during early abstinence have been conducted in male animals. However, research in females is beginning to reveal previously unknown sex-linked behavioural differences. In this line, recent studies using an ethanol-induced drinking programme have found a significant anxiogenic effect in male rats compared with female rats in the EPM (Sanz-Martos et al., 2023). Other studies have shown that, under normal physiological conditions, female rats tend to display less anxiety-like behaviour compared to males in this test (Börchers et al., 2022; Knight et al., 2021; Pavlova et al., 2020).

ApoAI potentiation by the peptide mimetic 4F of HDL pre-treatment did not appear to modulate the disinhibition/impulsivity effect observed in animals after IAC. However, other type of ApoA, such as ApoAIV, has shown to be related to stress responses since ApoAIV ko mice showed an anxiety-like behaviour. These anxiogenic effects appear to be strain-dependent, since they have been observed in 129X1/SvJ mice but no in C57BL6J mice (Packard et al., 2017), so more research is needed to completely rule out an ApoAI involvement.

Even though we observed a disinhibited-like behaviour in EPM after IAC, we did observe anxiety-like behaviour in OFT, where ethanol-treated females showed a decrease in the number of entries to the inner zone and in the time spent there in comparison to control groups, as well as a decrease in the number of grid crossings and rears. These parameters are indicative of anxiety-like behaviour after IAC. Although this test has been historically used to explore locomotor activity in rodents, it is frequently utilised in the context of anxiety-like behaviour research (Campbell et al., 2024). It is conceivable that, in view of these results, these two tests may not measure exactly the same behaviour, or that their use may need to be differentiated in the case of females, which may be more sensitive to the effects of one or the other. Sex differences in behaviour after LPS administration have been reported in middle-aged mice, where female showed higher sickness behaviour in the OFT than males and increases in circulating pro-inflammatory cytokines (Dockman et al., 2022). Interestingly, previous studies have shown that

control female animals typically exhibit greater locomotor/exploratory activity in the OFT, spending more time in open zones and displaying lower anxiety-like behaviours (Börchers et al., 2022; Knight et al., 2021; Pavlova et al., 2020).

Although we did not observe effects of HDL pre-treatment in the previous variables in the EPM, we found that both HDL groups showed a significant decrease in the number of rears versus control animals in the OFT, which is somehow indicative of anxiety-like behaviour due to HDL pre-treatment. This effect has previously been observed in male mice when HDL was administered alongside LPS (Radford-Smith et al., 2023), accompanied by a reduction in grid crossings. Surprisingly, this behaviour was also observed in female controls that underwent the HDL pre-treatment, which is in line with the results obtained in the expression of pro-inflammatory genes in the brain. These results provide support for the hypothesis that exogenous HDL administration may promote an inflammatory state in control animals.

Behavioural tests have been performed using methods widely supported by the literature. However, since many of these tests have been validated primarily on male rodents, it is challenging to determine whether the results accurately reflect the parameters they aim to measure. Thus, researchers are testing new validated tests in females, such as the Acoustic Startle Response (ASR) test, which might be a better fit in modelling female anxiety-like behaviour. ASR is considered a measure of anxiety that is independent of locomotion, as it relies on reflexive skeletal muscle contraction in response to an aversive acoustic stimulus (Börchers et al., 2022). Furthermore, estrogen's influence on anxiety-like behaviour has been reported as both anxiogenic and anxiolytic (Marcondes et al., 2001), or as not having any effect (Börchers et al., 2022; Scholl et al., 2019). In these behavioural studies we did not measure the estrous cycle for several reasons. Firstly, we had already conducted this procedure in Study I and had not found any alcohol-inducing changes or synchronization between the females in the experiment. Secondly, the procedure is time-consuming and collecting vaginal smears can be a very stressful process for the females involved. Thus, we aimed to prevent this variable to ensure the integrity of the behavioural tests. However, the possibility that the females in each experiment may have exhibited differing cycles cannot be dismissed.

Previous studies have shown an increase in **depressive-like behaviours** in male rodents after prolonged ethanol vapor exposure (Walker et al., 2010) and after ABD (Antón et al., 2017). In this study, IAC also induced a depressive-like behaviour in ethanol-treated females, since we observed a reduction in the climbing time and latency to immobility, as well as an increase in the immobility time. Depression and alcohol abuse have shown a high psychiatric comorbidity in humans (Boschloo et al., 2011; Conner et al., 2009; Fergusson et al., 2009). The TLR4 signalling

cascade has been shown to play a role in these behavioural alterations in males through the production of cytokines like TNF-α and IL-1β following its activation (Antón et al., 2017), although we did not observe gene expressions of these cytokines after IAC in females of this study. As mentioned before, these cytokines have been related to depressive-like behaviour even in healthy populations (Crews et al., 2017; Figueroa-Hall et al., 2020; García Bueno et al., 2016; Reichenberg et al., 2001).

We did not find a main effect of HDL in the Porsolt test on any of the parameters studied. However, we observed a significant decrease in the climbing time in the HDL control group versus the non-pretreated group, as well as a tendency to higher immobility time on average (not significant), suggesting a modest influence on depressive-like behaviour of HDL pretreatment. No main effects of 4F pre-treatment were observed in any of the parameters studied. Lipids have been identified as a promising class of peripheral biomarkers with the potential to facilitate quantitative diagnostic procedures (Parekh et al., 2017). Dyslipidemia and low HDL cholesterol, a known marker of cardiovascular disease, has been related to major depression in humans (A. L. Han, 2022; Khalfan et al., 2023; Melin et al., 2019; Penninx et al., 2013). However, recent studies have shown that high HDL levels can also be related to depression and cognition impairments (Y. H. Liu et al., 2024). A recent human study from our lab has shown that high levels of plasma ApoAI correlated with higher plasma LPS levels and worse cognition in AUD patients, and elevated plasma ApoAI could serve even as a potential AUD biomarker (Escudero et al., 2024). HDL was also closely related to depressive-like behaviour in mice after a high-fat diet (Yu et al., 2021). We did not measure plasma HDL or ApoAI levels in the behavioural study, but in Study I, in spite of no changes in the HDL profile after IAC, we did observe elevations in plasma ApoAI levels in ethanol-treated females versus controls. So, it is possible that elevated plasma levels of ApoAI contribute to this pro-depressive effect in ethanol-treated animals, although we cannot make direct comparisons due to animals come from different experiments. However, HDL also appears to affect control females, and this is in line with the apparent proinflammatory effect found in these animals and discussed in the previous section.

Interestingly, some authors have expressed concerns regarding the reliability of the Forced Swimming Test (Porsolt) for measuring depressive-like states in rodents. These concerns relate to the conditions (tank size, water temperature, body mass, etc) of the test and the interpretation of the results. Some changes have been proposed to mitigate the occurrence of false negative results, such as the use of larger tanks, hotter water, etc. With these changes, the authors hope to better differentiate active versus passive coping to stablish a depressive-like behaviour in rodents (Armario, 2021). However, these proposals are very recent and have yet to be broadly implemented within the scientific community. In this study, the test was performed under

conditions that have previously been described and are currently in wide use in order to reduce the number of variables.

The SPT was performed to ascertain the **motivational state** of abstinence, as it is a tool designed to evaluate anhedonic behaviour. Low saccharin preference is a well-established index of anhedonia in animal models (Scheggi et al., 2018) since sugars are high-value reinforcers for rodents. Some authors have observed that inflammatory stimuli, such as LPS, have been demonstrated to induce anhedonia until 30 hours after LPS injection (Sayd et al., 2015). Thus, in this study we show the effect of IAC and mimetic peptides. As mentioned before, animals were house individually after completion of the Porsolt test (which started 27h after the last ethanol binge) and saccharin preference was measured 36h, 54h and 60h after the last ethanol administration. Firstly, we did not find an ethanol main effect, although animals exposed to alcohol exhibit a trend to reduced preference for the sweet solution. The absence of significant group differences in saccharin preference during abstinence may indicate limitations in the sensitivity of the test as conducted, rather than the absence of an effect of alcohol abstinence on anhedonia. However, this may agree with the absence of effects on neuroinflammation found in females after ethanol binges. Moreover, females appear to prefer sweet solutions more than males (Grimm et al., 2022), which may also have masked the effects of ethanol, although there is a very scarce information about it in the literature. Interestingly, in the second study with 4F pre-treatment, we did find a significant ethanol main effect, with a reduction in the saccharin preference, indicative of anhedonia. Some authors did not find alcohol effects on anhedonia in mice after a drinking in the dark model (K. M. Lee et al., 2015). Others even observed that rats with a high preference for saccharin also consumed significantly more alcohol in a self-administration model than those with a low saccharin preference (Gosnell et al., 1992).

We also observed a sustained reduction in the saccharin preference after HDL pre-treatment, which could be indicative of an anhedonic state, which is again in line with the inflammatory results obtained with HDL pre-treatment both in control and ethanol group, as deficits in motivation have been related with inflammatory processes (Swardfager et al., 2016). However, 4F had no effect on this motivational test in female rats at the time points assessed. In humans, low levels of HDL have been associated with anhedonia (Loas et al., 2016). However, there is a lack of information about the effects of ApoAI potentiation and this core symptom of depression, so this should be taken into account in future research.

It is well-known that alcohol abuse not only affects emotional behaviours but also cognition in animals (Cippitelli et al., 2010; Marco et al., 2017) and humans (Escudero et al., 2024; Orio et al., 2018; Spear, 2018), having a significant impact on neuropsychological

functions, with memory being one of the most frequently studied areas (Maillard et al., 2020). In this study, to test **spatial memory** in our female rats, we performed the MWM test. As explained in the Methods section, prior to the probe trial, animals were trained during 4 consecutive days to find the platform, recording the latencies to reach it. Some authors have observed sex differences during the training sessions in the MWM in physiological conditions. For example, female subjects appear to display higher swimming speeds in the training sessions, without this implying faster platform location, suggesting that their navigation strategy was less directed than males (Zorzo et al., 2024).

In this study, IAC did not delay the animals' learning, and no differences were found between the groups after ethanol treatment. This is in line with previous studies after alcohol intermittent exposure (Schulteis et al., 2008) and this ABD model (Obernier et al., 2002b), although other authors have shown impaired spatial learning in Sprague-Dawley rats after ABD (Ji et al., 2018). Similar effects were observed in the test day, which was performed without the platform, with only a tendency to spend less time swimming around the quadrant due to alcohol consumption. Some authors have found spatial memory impairments after ethanol binges in Sprague-Dawley rats, showing a decrease in the time swimming in the quadrant and a decrease in the number of crossings (Ji et al., 2018). However, they performed the training sessions for the MWM only 3 days after the last ethanol administration, whereas in our study we performed it 6 days after it. It is important to note that memory impairments have been shown to be associated with chronic alcohol consumption (King et al., 2020; Santín et al., 2000). Thus, the current IAC protocol may not be sufficient to produce detectable changes in this cognitive domain.

A main effect of pre-treatments with HDL and 4F was identified in the training sessions in the third and fourth day, as animals pre-treated with these compounds required a longer duration on average to learn to locate the platform than those that were not, which could be indicative of impaired learning. Furthermore, a main effect of these pre-treatments was observed in the latency to reach the platform, with differences being found between saline and HDL/4F groups. Thus, controls pre-treated with HDL and 4F showed higher latency (indicating reduced memory function). These results may be related with the increase in the plasma LPS levels found after the HDL pre-treatment (discussed above). However, some studies have reported LPS effects on associative learning in a sex-dependent manner, as LPS administration showed to impair learning in males but not in females despite showing higher levels of proinflammatory cytokines (Patel et al., 2023). The present study revealed no beneficial effects of HDL or 4F pre-treatments in spatial memory, more to the contrary, with HDL or 4F control animals demonstrating an increase in the latency to reach the platform location, suggesting a memory impairment. However, some authors have demonstrated enhancements in spatial memory in the MWM in an AD mouse

model after 4F administration. In the mentioned study, the combination of 4F mimetic peptide and Pravastatin was observed to result in a reduction in the latency to locate the hidden platform, accompanied by an increase in the number of crossings and the duration of swimming in the quadrant in the MWM. These findings suggest an enhancement in cognitive function (Handattu et al., 2009). Further research is required to gain a more comprehensive understanding of the mechanisms underlying this behaviour.

Regarding the other kind of memory task that was analysed, IAC was shown inconclusive results, since alcohol treatment produced **recognition memory** impairment in one study and enhanced it in the other. Interestingly, in both cases alcohol appeared to have a main effect on long-term memory, suggesting that binge drinking may not be sufficient to cause short-term impairments in recognition memory, as it has been more closely related to chronic alcohol consumption. Firstly, in the initial group of female subjects involved in the experiment with HDL, alcohol only increased the latency to reach for the first time the novel object at 24h. This finding is consistent with the results of previous research, which demonstrated that chronic alcohol consumption impairs rats' performance on the object recognition test (Ciccocioppo et al., 2002; García-Moreno et al., 2002). However, recent studies in adolescent mice have shown that ABD did not alter the recognition memory, and no sexual differences were found (Bent et al., 2022). Unexpectedly, in the second batch of females involved in the experiment with 4F, IAC caused an increase in the DI and a reduction in the latency to approach the novel object, both observed at 24 hours. These results may be related with the apparent decrease in anxiety-like behaviour and the disinhibition observed in the EPM, since it is known that anxiety in rodents can reduce their natural curiosity, which may have a negative impact on their performance in memory tests (Atrooz et al., 2021). In addition, the disinhibition can be associated with elevated levels of locomotor activity following alcohol consumption, as previously outlined by other authors (Moya et al., 2022; Sanz-Martos et al., 2023), also affecting their performance.

While this IAC protocol seemed to have a detrimental impact only on long-term memory, we observed that HDL pre-treatment exhibited opposite effects for short- and long-term memories. On the one hand, it initially appears to improve the discrimination index in the short-term memory. On the other hand, it promoted a memory impairment in the long-term, decreasing the discrimination index. In contrast, the 4F pre-treatment did not demonstrate an impact on recognition memory. ApoAI has been linked to cognition in both animals (Handattu et al., 2009; Lefterov et al., 2010; Lewis et al., 2010) and humans (Escudero et al., 2024). Most of the research in this field has been conducted in animal models, with a focus on age-related neurodegenerative disorders, such as AD (Hottman et al., 2014). In these mentioned studies, ApoAI has been observed to aggregate with Aβ, contributing to the reduction of cerebral amyloid angiopathy and

decreased glial activation (Lewis et al., 2010). This suggests that the presence of ApoAI in the brain may offer a protective effect, potentially preserving cognitive functions, including memory. Indeed, other authors have also studied AD with ApoAI KO transgenic mice, observing that these animals that do not express ApoAI show memory impairments (Lefterov et al., 2010). However, little is known in the context of alcohol abuse. Surprisingly, contrary to the protective hypothesis just mentioned, a recent study from our lab has shown that higher levels of plasma ApoAI correlated with worse cognition in humans after alcohol abuse (Escudero et al., 2024), and preliminary results of the present studies also indicate a proinflammatory profile of HDL or 4F administration that has negative consequences even in control animals.

In any case, the results of this study suggest that intensive alcohol drinking interferes more with recognition memory than with spatial memory. It is important to note that both tests imply different brain structures, being the first more dependent on cortical structures (Morici et al., 2015) and the latter on the hippocampus (Broadbent et al., 2004). In this regard, it has been observed that cortical structures appear to be more vulnerable to the effects of alcohol toxicity (Fowler et al., 2014), which could also be related to the disinhibition observed, given the role of the PFC in impulse control. The present study has not examined the structure of the hippocampus, which is a limitation that must be considered in future research.

The use of the 4F peptide and HDL as therapeutics has been recently proposed for some pathologies due to beneficial effects found in preclinical settings. However, our study highlights the risk of repetitive use of these compounds both in an alcohol and control conditions regarding emotional/cognitive status. Given that ApoAI/HDL may bind to many endogenous components, it is unclear whether the mimetic molecules are binding these other targets at the doses and conditions tested. Further pharmacological studies are therefore required to provide more information on the use of ApoAI mimetics, as well as to establish a more effective administration pattern capable of eliciting a significant effect in animals. Anyhow, this Doctoral Thesis has provided a first insight into the binding of ApoAI and/or ApoB to bacterial components under IAC conditions in rodents, as well as the effects of ApoAI potentiation on neuroinflammation and the neurobehavioural alterations in female animals during ABD abstinence.

## LIMITATIONS OF THE STUDIES

We are aware of the limitations of these studies, some of which have also been outlined above. In the Study I, we observed notable sexual differences in the expression of apolipoproteins, lipoproteins, BELs, corticosterone, and other parameters in plasma. However, due to the independent upload and analysis of data from western blots for each sex, a comprehensive comparison between male and female subjects under both alcohol and control conditions was not possible. Despite this limitation, we were able to identify a differential affinity for apolipoproteins to bind LPS components in male and female ethanol-treated animals, which constitutes a significant result of this study. Altogether, these are undoubtedly novel results that open up doors in the field, but further studies are necessary to report results from a complete sexual difference perspective (i.e. sex differences in the expression levels in control animals, etc).

The decision to measure Lipid A, along with other components of LPS, was made based on its capacity to interact with lipoproteins such as HDL via its Lipid A backbone (Brandenburg et al., 2002). Research into how other parts of LPS, such as the Core, may interact with apolipoproteins, is a goal for future studies. Furthermore, the study did not elucidate whether the binding of Lipid A to apolipoproteins occurs peripherally, enabling the transportation of LPS to the brain, or whether it occurs within the brain or in both compartments. The cellular specific localization of the aggregates requires further investigation through additional flow cytometry and immunohistochemical studies.

The methodology employed in this thesis does not allow us to ascertain with certainty whether the LPS components were able to cross the BBB. However, our pilot study revealed a decrease in Lipid A in perfused animals but not in aggregates, which indicates that a small percentage of Lipid A observed is located outside of the BBB and other percentage may infiltrate it. This last finding aligns with a previous study using immunohistochemistry, which demonstrated that Lipid A-Apo bound forms not only infiltrated the brain but have been found in the blood-brain interfaces, such as tanycytes-like cells, ependymal cells and brain endothelial cells (Vargas-Caraveo et al., 2017), but further studies are necessary to provide definitive evidence. Other studies have shown partial permeability of LPS to the brain parenchyma in different conditions and according to the dose (Banks & Robinson, 2010) and by action of HDL or apolipoproteins (Martínez et al., 2021; Radford-Smith et al., 2023). In addition, it has been documented that LPS can alter BBB tight junction proteins and cross into the brain (Banks et al., 2015; Peng et al., 2021).Further investigation is required into the specific mechanism of the binding of LPS to different apolipoproteins, the cellular localization of the aggregates and its functional consequences under alcohol intoxication conditions. This will facilitate a more

comprehensive understanding of the crosstalk between alcohol, neuroinflammation and the neuroimmune response, as well as an exhaustive analysis from a sex/gender perspective.

According to our previous studies in males, to observe the neuroinflammatory effects of intensive alcohol drinking with this ABD protocol, it is necessary to obtain biological samples approximately three hours after the last dose of alcohol (Antón et al., 2017). The behavioural experiments lasted more time from the last binge and so another major limitation of this thesis is that the behavioural studies were necessarily conducted with different animal batches than those used in the biochemical studies, which difficult the extrapolation of results.

This thesis did not include behavioural studies with male subjects. Our primary objective was to explore the role of apolipoproteins in the context of alcohol abuse and, since the formation of aggregates after IAC was different in male and females, the pre-treatment used for behaviour had to be tested in a sex-specific way. Initially, studies that preceded this Thesis exclusively utilised male rats; however, we decided to explore for the first time female subjects as well, given the growing importance of incorporating both sexes in psychobiological and biomedical, among others, research. Due to time constraints and the paucity of literature on the subject, in addition to the sex-dependent results obtained in Study I, we focused on the behavioural studies in females, thus enhancing the novelty of this thesis. To complete these studies, it would be needed to potentiate ApoB in males and study neuroinflammation and behavioural alterations during abstinence, as well as obtaining a direct comparison of male and females, both in control and alcohol conditions, in behavioural tests.

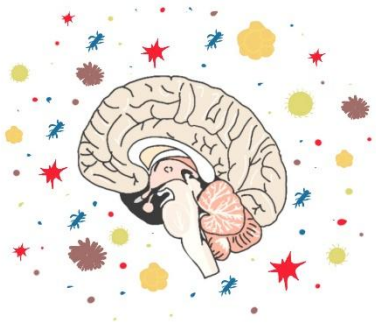
In the experimental design, HDL and 4F-peptide pretreatments were administered at the same dose and always 15 minutes before ethanol administrations, following a previous design for pharmacological treatments used in the research group (Antón et al., 2017, 2018). However, a dose-response curve would be required to ascertain the pharmacological effects of these compounds. Additionally, calories derived from alcohol were not replaced isocalorically with sucrose or other caloric-matched compounds in control groups, which received i.g. water, since our previous studies indicated no changes using isocaloric 5% dextrose with this four-day pattern of alcohol administration (Supplemental Information in Antón et al., 2017), although this aspect should be considered for chronic alcohol administrations.

Finally, plasma HDL molecules are not only composed by ApoAI but other Apos, such as ApoE, ApoM or ApoC, which makes these molecules less specific than the 4F mimetic peptide to potentiate ApoAI. Simultaneously, there is a paucity of literature concerning the potency of 4F, and studies with 4F and a scramble molecule will be required to test also neuroinflammatory

parameters as we have done with HDL. A scramble peptide (sc-4F, Ac-DWFAKDYFKKAFVEEFAK-$NH_2$) was tested in behavioural studies with the 4F as a control peptide for 4F (data not shown). It consists of a rearrangement of the amino acid sequence of 4F so that it could not form an amphipathic helix, losing its biological activity (Dai et al., 2010). However, the behavioural results obtained were inconsistent and with a high inter-subject variability, so these experiments should be repeated with this molecule in the future. This may also have limited to some extent the interpretation of the behavioural results. We used commercial HDL molecule coming from humans, as a commercially available compound. This may difficult the interpretation of results since the immunogenicity of human lipoprotein may differ from rodents. This issue has been previously addressed in the mentioned Radford-Smith and collaborators study, where lipoprotein-only controls were used for any cross-species immunogenicity of the human lipoproteins in mice, and they showed no difference in immune gene expression compared to the saline vehicle (Radford-Smith et al., 2023). It would also be interesting to conduct studies with ApoAI or ApoB KO mice/rats to test the effect of constitutively removing these apolipoproteins in our experimental conditions.

Despite these limitations, the findings of this Doctoral Thesis offer valuable insights into the health effects of a drinking pattern that is increasingly common yet less studied compared to chronic alcohol consumption. In addition, these novel results open up doors to new opportunities and advancements in the field of alcohol abuse and the link between apolipoproteins, neuroinflammation and behaviour in this context.

# 9. CONCLUSIONS



## CONCLUSIONS:

1. Lipid A, the endotoxic component of LPS, forms aggregates with ApoAI or with ApoB in the PFC of female and male animals, respectively, exposed to IAC, but not in the cerebellum. No brain aggregates were found with ApoE in any of these brain structures.

2. Lipid A and Core, the small components of LPS, and ApoAI, ApoB or ApoE levels were detected in their free (not bound) form in the PFC and cerebellum of control animals, but they were not altered after the intensive alcohol drinking either in males of females. However, the expression of ApoB receptor (LDLr) in the PFC and ApoE receptor (ApoER2) in the cerebellum were upregulated only in ethanol-treated males, with no changes in the expression of ApoAI receptor (SR-BI) either in males or females after IAC.

3. Sexual differences were found in plasma LDL and its main apolipoprotein, ApoB, with males having higher levels of this lipoprotein and apolipoprotein than females, disregarding of alcohol effects. Although no changes were observed in plasma HDL profile, its main apolipoprotein, ApoAI, was increased in ethanol-treated females in comparison to female controls, with no changes in males.

4. Males, but not females, reached binge levels (>0.08g/dL) 3h after the last alcohol binge in the IAC protocol used, and ethanol-treated females showed higher plasma corticosterone levels versus her controls, an effect not found in males.

5. Males and females exposed to IAC showed higher levels of plasma LBP, a marker of bacterial translocation and inflammation in the periphery. In the brain, protein levels of TLR4, a key component of the innate immune system and a sign of neuroinflammation, was found upregulated in the PFC of males that underwent IAC. This effect was not observed in females after IAC.

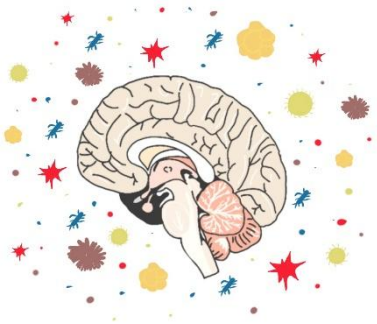
6. At the behavioural level, the results of the emotional assessment revealed signs of disinhibited-like behaviour in alcohol-treated females in the EPM. However, ethanol-treated females showed anxiety in the OFT, as well as depressive-like behaviour and anhedonia. In the cognitive assessment, a slight deficit in the long-term recognition memory, but no in learning or spatial memory, were observed in females that underwent IAC during early abstinence.

**7.** Repeated HDL administration promoted peripheral inflammation increasing plasma LPS levels both in control and ethanol-treated females. HDL also induced neuroinflammation in the PFC, observed by the increment of gene expression of pro-inflammatory cytokines (HMGB1, CXCL1, TNF-α) and the innate immunity receptor TLR4, disregarding of the ethanol effects.

**8.** Pre-treatments with HDL or the ApoAI mimetic peptide, 4F, before every binge did not prevent behavioural alterations after alcohol exposure in female animals. HDL, as unspecific pharmacological tool to potentiate ApoAI, produced learning deficits in the MWM, aggravated recognition memory, promoted anxiety-like behaviour and anhedonia in both experimental groups, and even aggravated spatial memory in control females. The 4F peptide, as a specific way to potentiate ApoAI, also produced learning impairments in the MWM in both ethanol-treated and control females and affected spatial memory only in control females.

In a whole, the results of this Doctoral Thesis suggest an association between apolipoproteins and bacterial products after IAC, dependent on sex, which may have implications for alcohol-induced neuroinflammation and neurobehavioral responses during early abstinence.

# 10. BIBLIOGRAPHY



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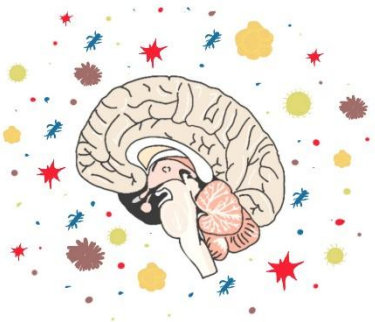
# 11. ANNEX



The main results of the Study I of this Doctoral Thesis are reported in the following publication:

**López-Valencia, L**., Moya, M., Escudero, B., García-Bueno, B., & Orio, L. (2024). Bacterial lipopolysaccharide forms aggregates with apolipoproteins in male and female rat brains after ethanol binges. Journal of Lipid Research, 65(3). https://doi.org/10.1016/j.jlr.2024.100509

JLR RESEARCH ARTICLE

# Bacterial lipopolysaccharide forms aggregates with apolipoproteins in male and female rat brains after ethanol binges

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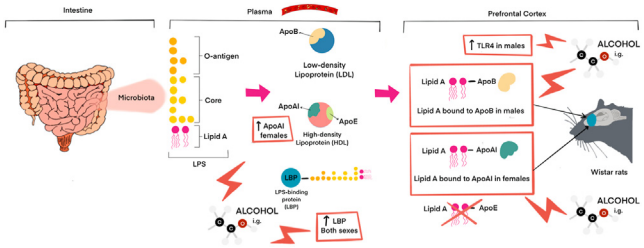
**Abstract** **Alcohol binge drinking allows the translocation of bacterial lipopolysaccharide (LPS) from the gut to the blood, which activates the peripheral immune system with consequences in neuroinflammation. A possible access/direct signaling of LPS to/in the brain has not yet been described under alcohol abuse conditions. Apolipoproteins are compounds altered by alcohol with high affinity to LPS which may be involved in its transport to the brain or in its elimination. Here, we explored the expression of small components of LPS, in its free form or bound to apolipoproteins, in the brain of female and male rats exposed to alcohol binges. Animals received ethanol oral gavages (3 g/kg every 8 h) for 4 days. LPS or its components (Lipid A and core), LPS-binding protein, corticosterone, lipoproteins (HDL, LDL), apolipoproteins (ApoAI, ApoB, and ApoE), and their receptors were measured in plasma and/or in non-perfused prefrontal cortex (PFC) and cerebellum. Brain LipidA-apolipoprotein aggregates were determined by Western blotting and confirmed by co-immunoprecipitation. In animals exposed to alcohol binges: 1) plasma LPS-binding protein was elevated in both sexes; 2) females showed elevations in plasma ApoAI and corticosterone levels; 3) Lipid A formed aggregates with ApoAI in the female PFC and with ApoB in males, the latter showing Toll-like receptor 4 upregulation in PFC but not females. These results suggest that small bacterial components are present within the brain, forming aggregates with different apolipoproteins, depending on the sex, after alcohol binge intoxications. Results may have implications for the crosstalk between alcohol, LPS, and neuroinflammation.**

**Supplementary key words** alcohol • binge drinking • LPS • Lipid A • apolipoprotein • TLR4 • ApoAI • ApoB • neuroinflammation • sex-differences




Alcohol binge drinking (ABD) is one of the most widespread patterns of alcohol consumption worldwide among adolescent people. It consists of a heavy intake of alcohol that brings blood ethanol concentration (BEC) to ≥ 0.08 g/dl in a short period of time. It means five or more alcoholic beverages for males and four or more in females in approximately two hours (1, 2). This consumption produces acute and chronic effects, which may affect organs such as the brain, gut, and liver. ABD affects the structure and/or function of the central nervous system (CNS), including loss of white matter and behavioral alterations such as cognitive impairment. Anyone can be susceptible to its deleterious effects, especially adolescents, due to their immature prefrontal cortex (PFC), which is responsible for executive functions and impulse control (3–5). Many of these changes during adolescence may remain into adulthood in rat models (6).

Other organs also affected by ABD are the gut and the liver. ABD induces gut dysbiosis, promoting an increase in *Gram*-negative bacteria, gut inflammation, and disruption of the intestinal barrier, allowing the translocation of bacteria to the mesenteric lymph nodes and

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 https://doi.org/10.1016/j.jlr.2024.100509

bacterial components, such as lipopolysaccharide (LPS), to the systemic circulation, an effect known as leaky gut (7–11).

These events have been proposed as possible triggers of the well-known peripheral inflammatory and neuroinflammatory response induced by alcohol, activating the innate immune system by increasing the TLR4 receptor, inducing the translocation of the nuclear factor *kappa* B transcription factor to the nucleus, and promoting the expression of pro-inflammatory mediators such as cytokines, chemokines, and high-mobility-group box protein 1, which are related to cellular damage (6, 12, 13). Activation of the TLR4 proinflammatory pathway has been directly related to some behavioral disturbances during abstinence, such as depressive-like behavior, anxiety, or anhedonia (14, 15).

As mentioned above, LPS is a large component of the outer membrane of *Gram*-negative bacteria (16). It consists of three different parts attached to each other: Lipid A, a glycolipid domain that determines toxicity and inflammation mainly by activating TLR4; the core, a short chain of sugar residues; and the O-antigen, a highly variable polysaccharide moiety (17, 18). Under physiological conditions, low circulatory levels of LPS have been detected in the plasma of animals (19) and humans (17). LPS-induced cellular responses are the net result of the interaction of LPS with various plasma components, such as soluble cluster of differentiation 14 (CD14), LPS-binding protein (LBP), phospholipid transfer protein, and membrane receptors, such as membrane-bound CD14 and TLR4. Balanced cellular responses are essential for the host defense against bacterial infections. However, if large amounts of LPS are present in the circulation, an excessive cellular response can be deleterious for the host, and therefore, endotoxin-inactivating processes are of extreme importance (18). There are several routes of LPS detoxification in the circulation, but the most relevant is by its incorporation into lipoproteins (20).

Lipoproteins can be classified according to their density as very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). Their protein part is composed of apolipoproteins (Apos) (type AI or E in HDL (20), type B in LDL (21), and type B100 or E in VLDL) (22). Apolipoproteins transport lipids in polar water-based solutions such as blood, cerebrospinal fluid, and lymph. Apolipoproteins interact with membrane lipoprotein receptors and lipid transfer proteins to regulate lipoprotein uptake and clearance and serve as enzyme cofactors for enzymes implicated in the metabolism of lipoproteins (23). There is increasing evidence supporting the idea that apolipoproteins are involved in functions beyond cholesterol transport (24), such as the detoxification of bacterial LPS as actors of the innate immune system (25). The first step in LPS detoxification is its binding to HDL. Once LPS is bound to HDL, its inflammatory activity is reduced. Although HDL may protect initially, LPS should be moved to LDL because the `buffering' capacity of HDL is quickly exceeded (26). In a second step, acute phase transfer proteins LBP and phospholipid transfer protein efficiently redistribute LPS from HDL to other lipoprotein subclasses, such as LDL and VLDL, in a time-dependent manner (27). Pathogen lipids incorporated into LDL and VLDL are then cleared by the liver via the LDL receptor (LDLr) (and possibly other lipoprotein receptors such as VLDL receptor, ApoE receptor 2 (ApoER2), and scavenger receptor class B type 1 (SR-BI)) and secreted in bile (19).

One controversial question is how peripheral LPS can stimulate the activation of the innate immune response in the brain (28). Structures lacking the blood brain barrier (BBB), such as the circumventricular organs, and other blood–brain interfaces, such as the choroid plexus and the meninges, rapidly respond to pro-inflammatory stimuli by LPS (29). The direct infiltration of LPS into brain tissue has been questioned (30), but several, not exclusive between them, humoral/cellular routes have been proposed: 1) by means of cytokine signaling through the vagus nerve; 2) increased BBB permeability; 3) vascular prostaglandin effects; 4) leukocyte infiltration. We have recently shown that small components of *Escherichia Coli* LPS (Lipid A and Core) may infiltrate the brain under physiological conditions bound to apolipoproteins (31). Specifically, we observed LipidA-apolipoprotein aggregates in tanycytes-like cells (interface between blood and CSF) and ependymal cells in circumventricular organs, but we also observed positive staining in brain-endothelial cells, for example, in the hippocampal commissure, and even in astrocytes of the medulla oblongata (31). Astrocytes and tanycytes in the circumventricular organs of the brain are known to be crucial for initiating the LPS-induced inflammatory responses via TLR4 (32). Interestingly, we also observed minimal presence of Lipid A aggregates in the PFC of perfused animals under physiological conditions.

It is not clear at present whether the binding of LPS to apolipoproteins is involved in the transport or signaling of LPS to the brain or it is a mechanism for endotoxin detoxification. The first would indicate a direct mechanism for LPS signaling in the brain, with consequences in neuroinflammation; the latest would indicate an attempt of the body to neutralize alcohol-induced neuroinflammation by forming LPS-apolipoprotein aggregates. Whatever the functional consequences, an interesting preliminary research question is to investigate the state of LPS components and apolipoproteins in the brain of animals exposed to alcohol intoxications, since alcohol abuse disrupts the BBB and induces neuroinflammation (33).

In the present study, we aimed to explore whether there are differences in the expression of small LPS components, apolipoproteins, or their aggregates in

animals exposed to alcohol binge intoxications compared with controls. We explored two brain structures affected by alcohol, such as the PFC and the cerebellum.

We hypothesized that H1) animals which undergo alcohol binge intoxications show more expression of small LPS components (Lipid A or Core) or their aggregates with apolipoproteins in the brain than control animals; H2) H1 could differ in male and female animals exposed to alcohol binge intoxications and in different brain regions.

## MATERIALS AND METHODS

### Animals

Forty-four Wistar rats (Envigo©, Barcelona, Spain) aged six weeks were used across all experiments. Upon arrival, females weighed 160–220 g and males 180–230 g and were housed in different isolated rooms. Animals were housed in groups of 2–3 per cage and maintained at constant conditions of temperature (21 ± 1°C) and humidity (59 ± 10%) under a 12 h dark-light inversed cycle (lights on at 8:00 p.m.) with free access to food and water. Animals were habituated to these conditions for 11 days before the experiments, at which time they were handled gently to acclimate to the experimenters and gavage procedure.

All procedures were approved and adhered to the guidelines of the Animal Welfare Committee of the Complutense University of Madrid (Ethical approval reference: PROEX 312/19) following European legislation (2010/63/EU).

### Experimental design and ethanol intoxication procedure

Animals were randomly assigned to control and experimental groups: male control group, male ethanol group, female control group, and female ethanol group. Rats received intragastric (i.g.) ethanol or water three times per day using specific cannulae (16-G needle, Fisher Scientific, Waltham, MA), following a standard paradigm of a 4-days binge alcohol intoxication protocol previously used by our group (10, 15) and by others (34). An additional control group of animals was used in a pilot study to compare the effects in saline perfused versus nonperfused animals prior brain extraction.

Ethanol solutions were prepared daily from 96% ethanol stock diluted in water, and body weights were measured daily 120 min before the beginning of the i.g. gavage. Female and male ethanol-treated rats received an initial loading dose of 5 g/kg in a 30% solution (w/v) and then a maximum of 3 g/kg for additional doses (Supplemental Table S1). This repeated binge-pattern ethanol paradigm maintained relatively constant intoxicating BEC in a range of sedation/ataxia according to the 6-point behavioral ethanol intoxication scale (35). In this study, the average dose of ethanol per rat was 8.73 g/kg/day.

The female reproductive cycle was controlled during the experiment by collecting vaginal smears once a day at the same time of the day to reduce variability. Vaginal secretions were collected with a plastic pipette filled with normal saline (NaCl 0.9%) by introducing the tip gently into the rat vagina, and the vaginal fluid was placed on different glass slides and immediately examined under a light microscope. Estrous cycle phases were determined by observation of cell types in the entire smear (36) by using a Nikon Japan microscope (Nikon Instruments, Inc., Melville, NY) (Supplemental Fig. S1).

### Tissue and plasma collection

Following an alternation of the four experimental groups, samples were taken three hours after the last ethanol administration prior to administration of a lethal dose of sodium pentobarbital (320 mg/kg, i.p., Dolethal®, Spain). Blood was collected by cardiac puncture using ethylenediaminetetraacetic acid (molecular weight 452.24 g/mol, pH 7.2) as an anticoagulant, and then animals were decapitated. Blood samples were centrifuged at 4°C for 15 min at 2,000 g for plasma fraction collection, which was stored at −80°C until assay. Brains were rapidly isolated from the skull, discarding blood vessels and meninges, and the PFC was excised and frozen at −80°C until assayed.

Using this experimental protocol for samples collection, we reproduce real conditions as much as possible, since the blood-flow in the brain vessels could be an important source of molecules that drive the alcohol-induced neuroimmune response. In this sense, it has been proposed that circulating factors greatly retard the interaction of LPS with the BBB, and the luminal binding of LPS is enhanced when those factors are removed (30). Although washing the vascular space of the brain does not reproduce real conditions, we have used this complementary experimental approach in a pilot study using an independent group of animals that were intracardially perfused with sterile physiological saline solution (0.9% NaCl) prior brain collection to check the contribution of PFC blood flow in the observed effects.

### Western blot analysis

Brain samples were homogenized by sonication in PBS (pH = 7.4) mixed with a protease inhibitor cocktail (Complete, Roche®, Madrid, Spain) at a dilution of 1:3 (w/v), followed by centrifugation at 13,000 rpm at 4°C for 10 min. Protein levels were measured and adjusted by Bradford's method, and homogenates were mixed with Laemmli simple buffer (Biorad®, Alcobendas, Madrid, Spain) containing β-mercaptoethanol (50 µl/ml of Laemmli) to obtain a final concentration of 1 mg/ml. Proteins were separated by an electrophoresis gel, blotted onto nitro-cellulose membranes (Amersham Ibérica®, Madrid, Spain) with a semidry transfer system (Bio-Rad®, Madrid, Spain), incubated with specific primary and secondary antibodies (Supplemental Table S2), and revealed by using a chemiluminescence system (ECL™-kit) (Amersham Ibérica®, Madrid, Spain). Autoradiographs were quantified by densitometry (NIH ImageJ® software, National Biosciences, Lincoln, Nebraska) and expressed as optical density (O.D.). In all Western blot analyses, the housekeeping β-actin protein was used as a loading control. Every blot contained different samples per group, and two blots were run in separate assays. The results represent the average of two technical replicates.

To maximize the analysis of multiple proteins with a limited tissue, the membranes in western blots were cut, and each small membrane was incubated with the antibody of interest. The blots are represented in the images as they were loaded (vertical distribution before cutting the membrane). When necessary, a stripping procedure was performed, as indicated in the representative blots of the figures. The samples of Western blot were loaded separately for male and female groups, so no direct comparation between sexes was done in these analyses.

In a pilot study, we demonstrate that Lipid A is bound to different proteins, all of which are of interest in our study. This binding activity was visualized by both Western blot and coimmunoprecipitation (co-IP) procedures (next section). The analysis of bound forms to Lipid A by Western blot was performed first by incubation of samples with the antibody against the specific protein of interest (i.e., ApoAI). Then, a stripping procedure was performed, and the membranes were incubated with the antibody against Lipid A, which shows a band with a similar molecular weight to the protein of interest (note that free Lipid A weighs ~10 kDa), indicative of the [Lipid A-protein] complex (which has also been demonstrated by co-IP procedures). The results of the binding between Lipid A and each apolipoprotein were expressed as the quantification of the bound form normalized by the total amount of the specific protein/apolipoprotein in this brain area.

## co-IP procedure

Co-IP is a biochemical method to precipitate a complex using target-specific antibodies. In this study, co-IP was used to study LipidA-Apo binding (protein−protein interaction). The Lipid A antibody was first used to immunoprecipitate, and then it was immunoblotted using specific ApoAI or ApoB antibodies (Apo E was not used because no colocalization of Lipid A and ApoE was found in Western blotting). The signal obtained in the co-IP means that ApoAI or ApoB is immunoprecipitated in Lipid A, confirming the binding of Lipid A to ApoAI or ApoB.

Co-IP was performed based on a previously published protocol (37, 38). Brain samples were mechanically homogenized using 5 mm stainless steel beads in a TissueLyser LT (Qiagen®, Hilden, Germany) with 1 ml of 50 mM Tris buffer (pH = 7.4) mixed with a protease inhibitor cocktail for each brain tissue sample. The frequency used was 50 oscillations for 2 min 3 times, followed by centrifugation at 1 000 g for 10 min at 4°C. The supernatants were collected and centrifuged at 12,000 g for 30 min at 4°C. Then, the pellets were resuspended in Tris buffer at a dilution of 1:750 (w/v). Protein levels were measured and adjusted by Bradford's method, and homogenates were mixed with Tris buffer to obtain a final concentration of 4 mg/ml.

To obtain concentrated samples from small amounts of tissue and due to the low signal intensity obtained after co-IP during the first trials, a sample pooling of two biological replicates in a group was performed. All samples were centrifuged at 12,000 g for 30 min at 4°C, and pellets were resuspended in 200 µl of RIPA buffer (R0278, Sigma−Aldrich®, Madrid, Spain) containing protease inhibitor cocktail and incubated for 30 min with constant rotation at 4°C. Samples were centrifuged at 12,000 g for 30 min at 4°C, and supernatants were collected in Eppendorf tubes containing 10 µl of Lipid A antibody (Supplemental Table S3) and incubated overnight with constant rotation at 4°C to allow the formation of immune complexes. Twenty-five microliters of Protein A Agarose resin (P3476, Sigma−Aldrich®, Madrid, Spain) was added to the samples and incubated with constant rotation for 2 h at 4°C to collect the immune complexes. The resin was washed three times by centrifuging at 10,000 g for 1 min at 4°C. Resins were first resuspended in 200 µl of RIPA buffer and, in the last wash, with Laemmli simple buffer containing β-mercaptoethanol, and they were analyzed by SDS−PAGE and immunoblotted using ApoAI or ApoB antibodies (Supplemental Table S2).

## BEC determination

Ethanol levels in plasma samples were measured by the commercial Enzychrom TM Ethanol Assay Kit ECET-100 (BioAssay Systems®, Hayward, CA) according to the manufacturer's protocol. The absorbance of each well was measured at 570 nm using a ThermoMax microplate reader (Molecular Devices®, Ramsey).

## Plasma corticosterone levels

Plasma corticosterone levels were determined by a colorimetric competitive enzyme immunoassay kit (Catalog No. ADI-900-097, Enzo Life Sciences®, Lauren, Switzerland) following the manufacturer's instructions. Standards and plasma samples were assayed in duplicate. Absorbance was measured at 405 nm using a ThermoMax Microplate reader (Molecular Devices®, Ramsey, USA). Calculated values are expressed as nanograms of corticosterone per milliliter (ng/ml).

## Plasma LPS determination

Plasma LPS levels were determined using a commercially available kit based on ELISA following the manufacturer's instructions (Hycult Biotech®, Uden, The Netherlands). This test is based on the ability of the endotoxin to cause intravascular coagulation in the American horseshoe crab, Limulus polyphemus. This endotoxin causes an opacity and gelation in Limulus amebocyte lysate, producing an enzymatic reaction and a yellow color. LPS was measured at 450 nm (Molecular Devices®, Ramsey). The results were obtained as endotoxin units per mL (EU/ml) and expressed as a percentage of control values.

## Determination of apolipoproteins

ApoAI, B, and E in plasma samples were measured using the sandwich-ELISA principle with a commercial assay kit (Catalog No. E-EL-R3029, E-EL-R1218, E-EL-R1230, respectively, Elabscience Biotechnology® Inc.) following the manufacturer's instructions. The absorbance was measured at 450 nm using a ThermoMax microplate reader (Molecular Devices®, Ramsey).

## HDL and LDL levels

HDL and LDL levels were measured in rat plasma using commercially available sandwich enzyme immunoassays (SEB006Ra-96T and SEB107Ra-96T, respectively, Cloud-Clone Corp.®, TX). The final concentrations of HDL and LDL in the samples were determined by comparing the O.D. of the samples to the standard curve by measuring the color change spectrophotometrically at 450 nm wavelength.

## Statistical analyses

All data are expressed as the mean ± S.E.M. Data from ELISA kits were analyzed using a 2-way ANOVA, comparing the factors [alcohol/water] versus sex [male/female], when normality was verified; otherwise, a Kruskal-Wallis test was used. *Post hoc* comparisons (Bonferroni) were performed in case of significant interaction between factors. Homoscedasticity was checked by Barlett's test, and data were transformed (sqrt, $\log_{10}$) when appropriate. Data from western blots of each sex were analyzed independently, comparing alcohol-treated animals versus controls by using the

parametric Student's *t* test or the nonparametric Mann–Whitney test, due to the samples from each sex were loaded in different blots. The outliers were analyzed using Grubbs' test. Correlations were assessed by Pearson's and linear regression analyses. A $P$ value < 0.05 was set as the threshold for statistical significance in all statistical analyses. All data were analyzed using GraphPad Prism version 8.01 (GraphPad Software, Inc., La Jolla, CA).

## RESULTS

### Plasma LPS, LBP, and Apos (AI, B, and E) levels in male and female ethanol-intoxicated and control animals

The levels of the different mediators were measured 3 h after the last ethanol i.g. administration and results analyzed using 2-way ANOVA in order to study differences between the four groups, including sexual differences. LPS was detectable in plasma in control animals both in male (0.491 ± 0.087 EU/ml) and female rats (0.612 ± 0.079 EU/ml). After 2-way ANOVA analyses, we did not find significant changes in plasma LPS levels (no interaction between factors: $F_{(1, 29)} = 0.01027$, $P = 0.9200$), but we observed an overall ethanol effect near of significance in both ethanol-treated groups (Fig. 1A; $F_{(1, 29)} = 3.441$, $P = 0.0738$) with no sexual differences ($F_{(1, 29)} = 0.4960$; $P = 0.4869$, respectively).

LBP was also detectable in plasma in all the experimental groups, and it was elevated in the ethanol-treated groups versus control groups (Fig. 1B; 2-way ANOVA, overall alcohol effect: $F_{(1, 28)} = 6.436$, $P = 0.0170$), disregarding of sex ($F_{(1, 28)} = 0.3616$; $P = 0.5525$; no interaction $F_{(1, 28)} = 0.8090$; $P = 0.3761$).

Regarding ApoAI levels in plasma (Fig. 1C), control males showed similar basal levels (529.15 ± 41.53 µg/ml) than control females (498.44 ± 62.68 µg/ml). 2-way ANOVA reported a significant interaction between ethanol and sex (Fig. 1C; $F_{(1, 31)} = 4.376$, $P = 0.0447$) with an overall ethanol effect ($F_{(1, 31)} = 4.489$; $P = 0.0422$) and a sex effect near of significance ($F_{(1, 31)} = 3.368$, $P = 0.0761$). *Post hoc* comparisons revealed that plasma ApoAI was elevated in female alcohol-treated rats versus female controls and versus alcohol-treated males (Fig. 1C, $P < 0.05$ in both cases).

Plasma ApoB (Fig. 1D) showed an overall sex effect ($F_{(1, 30)} = 16.40$, $P = 0.0003$) and an interaction between factors ($F_{(1, 30)} = 4.762$, $P = 0.0371$) after 2-way ANOVA. *Post hoc* test revealed a basal sexual dimorphism with control females showing lower levels of plasma ApoB (684.19 ± 1.18 µg/ml) than control males (1206.68 ±

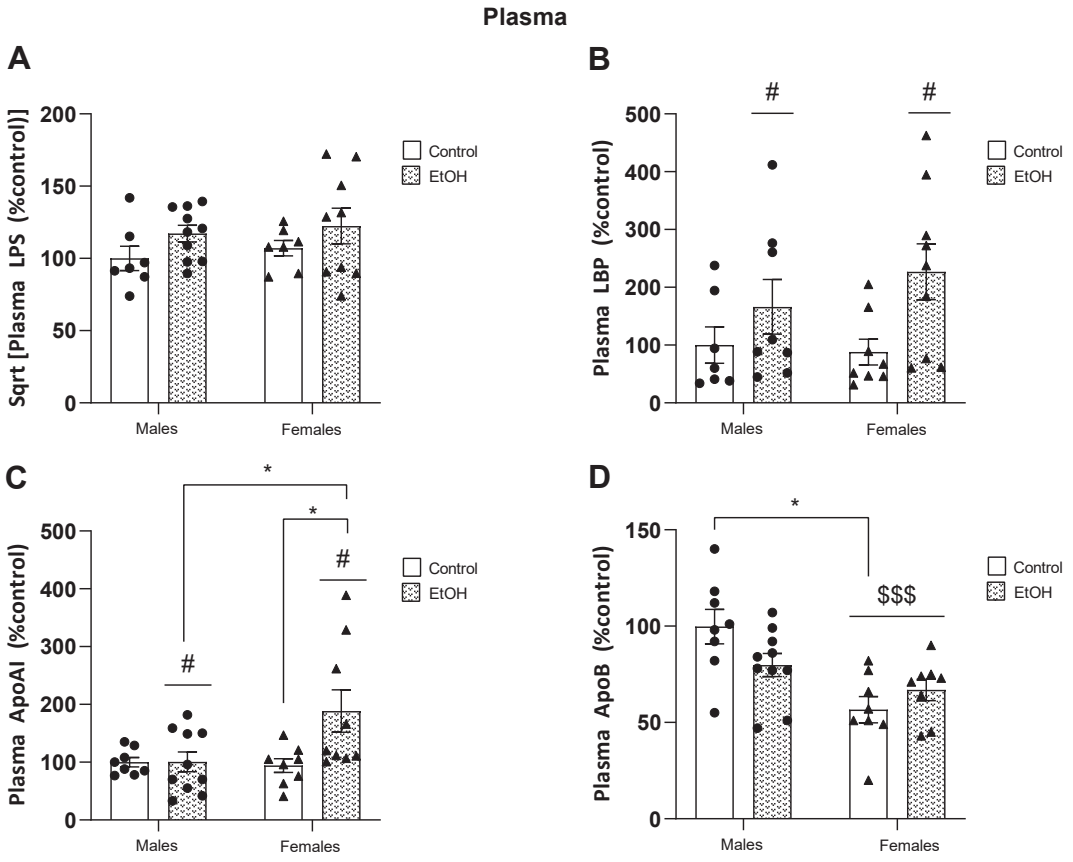


**Fig. 1.** Expression of LPS, LBP, and apolipoproteins in the plasma of male and female ethanol-treated and control animals. A: Plasma LPS levels. B: Plasma LBP levels. C: Plasma ApoAI. D: Plasma ApoB. No detectable levels for plasma ApoE. All data are expressed as mean ± S.E.M. Statistical analysis: 2-way ANOVA: overall effect of ethanol: $^{\#}P < 0.05$; overall effect of sex: $^{\$\$\$}P < 0.001$; interaction between factors (alcohol/sex), followed by Bonferroni *post hoc* test: $^{*}P < 0.05$. LBP, LPS-binding protein; LPS, lipopolysaccharide.

107.29 μg/ml) but no specific effect of alcohol ($F_{(1, 30)}$ = 0.4913, $P$ = 0.4888, n.s.) (Fig. 1D).

ApoE levels in plasma were under detection limits in ELISA in all experimental and control groups tested.

### Plasma HDL and LDL, corticosterone, and BECs in male and female animals

Since each Apo studied in the section before is mainly incorporated into lipoproteins of different densities and according to the LPS transport or detoxification theories explained in the introduction, we quantified the plasma levels of HDL (which incorporates mainly ApoAI and ApoE) and LDL (which incorporates ApoB).

The results of plasma HDL and LDL are shown in Table 1. A 2-way ANOVA found no interaction between factors for HDL ($F_{(1, 31)}$ = 0.6047, $P$ = 0.4427) and no overall alcohol ($F_{(1, 31)}$ = 0.7839, $P$ = 0.3828) or sex ($F_{(1, 31)}$ = 0.7870, $P$ = 0.3819) effects. Regarding LDL, the 2-way ANOVA indicated an overall effect of sex ($F_{(1, 31)}$ = 9.449, $P$ = 0.0044) and no alcohol effect ($F_{(1, 31)}$ = 2.626, $P$ = 0.1152) or interaction ($F_{(1, 31)}$ = 0.9269, $P$ = 0.3431), revealing a basal sexual dimorphism with males showing elevated plasma LDL than women (Table1).

We additionally checked BECs and corticosterone in males and females (Table 1). The mean BECs achieved 3 h after ethanol administration in males were within the binge drinking definition, whereas females had lower BECs (<80 mg/dl; Table 1, Student's $t$ test, $P$ < 0.05). However, regarding corticosterone levels, an interaction between factors was found ($F_{(1, 30)}$ = 10.01, $P$ = 0.0036) with overall ethanol and sex effects ($F_{(1, 30)}$ = 6.165; $P$ = 0.0188; $F_{(1, 30)}$ = 7.282; $P$ = 0.0113, respectively). *Post hoc* comparisons revealed that corticosterone was not altered during experimental conditions in males but it was increased in female alcohol-treated animals versus female controls ($P$ < 0.01). Estrous cycles in female rats were recorded during the experiment, and they are shown in the Supplemental Information (Supplemental Results 2.1, Supplemental Table S4 and Supplemental Fig. S2).

### Detection of LPS components (Lipid A and Core) in the brains of male and female alcohol-intoxicated and control animals

The components of LPS, Lipid A and Core, were measured in nonperfused PFC (**Fig. 2**) and cerebellum (Supplemental Fig. S3) of male and female animals treated with alcohol binges and their control groups. Interestingly, both Lipid A (free form, see results Screening study of the binding of Lipid A to different molecules in the PFC: Pilot study with TLR4) and Core were detectable and measurable within the brain (including cerebral blood flow) of males and females in both experimental groups and in both brain structures. Samples in western blots were uploaded independently by sex and, thus, results are graphed as percentage of change over each respective control group and analyzed accordingly.

Figure 2 shows the expression of LPS components in the PFC of male (upper panel) and female (lower panel) animals. There were no significant differences in the expression of Lipid A or Core levels (Fig. 2A, B; $t_{(16)}$ = 0.3174, $P$ = 0.7551; $t_{(16)}$ = 0.4200, $P$ = 0.6801, respectively) between the ethanol and control groups in males. Representative blots are shown in Fig. 2C.

In females, there were no changes in Lipid A and Core between the control and alcohol-treated groups (Fig. 2D, E; Mann–Whitney U = 21; $P$ > 0.05, n.s. ; $t_{(15)}$ = 0.7107, $P$ = 0.4881, respectively). Representative blots are shown in Fig. 2F.

The results in the cerebellum were not significant between groups and are shown in the Supplementary Results (Supplemental Fig. S3).

### Screening study of the binding of Lipid A to different molecules in the PFC: Pilot study with TLR4

After studying the expression of Lipid A and Core in the PFC in their free forms, our goal was to investigate the possible binding of these elements to different apolipoproteins within the brain. We chose Lipid A (the LPS domain considered endotoxin) for this colocalization study.

TABLE 1. Plasma HDL and LDL, corticosterone, and BELs

| | Males | | Females | | | |
|---|---|---|---|---|---|---|
| | Control | EtOH | Control | EtOH | Student's $t$ test | 2-way ANOVA |
| HDL (μg/ml) | 517.23 ± 43.30 | 415.08 ± 63.69 | 414.98 ± 60.73 | 408.36 ± 68.51 | – | n.s. |
| LDL (μg/ml) | 171.10 ± 6.27 | 138.20 ± 15.24 | 119.70 ± 12.18 | 111.32 ± 13.02 | – | Overall sex effect: $^{\$\$}P$ < 0.01 |
| Corticosterone (ng/ml) | 186.53 ± 38.74 | 156.11 ± 15.81 | 165.74 ± 22.97 | 418.05 ± 71.83*$^{\&}$ | –– | Overall alcohol effect: $^{\#}P$ < 0.05<br>Overall sex effect: $^{\$}P$ < 0.05<br>Interaction (*post hoc* test): *$^{\&}P$ < 0.05 |
| BEL (mg/dl) | – | 101.52 ± 19.61 | – | 56.95 ± 17.09* | $P$ = 0.0491 | – |

No differences between males and females were detected in plasma HDL. An overall effect of sex was found in LDL levels, with higher levels in males. There was an interaction between alcohol and sex in the levels of corticosterone: alcohol-treated females had higher corticosterone levels than the female controls (Bonferroni *post hoc* test: *$P$ < 0.05) and alcohol-treated males (Bonferroni *post hoc* test: $^{\&}P$ < 0.05). BEL (blood alcohol levels achieved 3 h last ethanol gavage) differed between male and female animals (Student's $t$ test, *$P$ < 0.05). Data are shown as the mean ± S.E.M. Statistical analysis: 2-way ANOVA: overall effect of ethanol: $^{\#}P$ < 0.05; overall effect of sex: $^{\$\$}P$ < 0.001; interaction between factors (alcohol/sex), followed by Bonferroni *post hoc* test: *$P$ < 0.05.

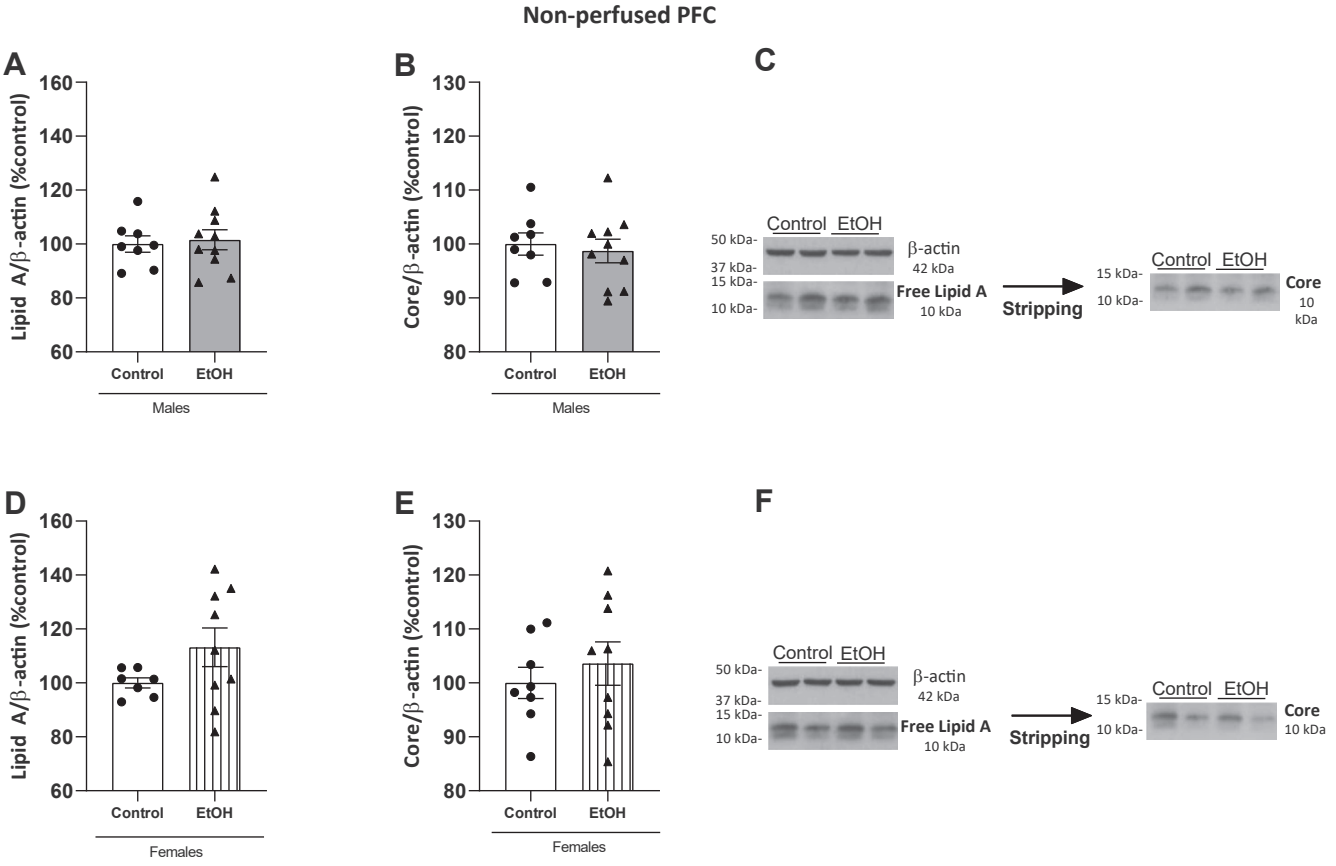


**Fig. 2.** Detection of the LPS components Lipid A and core in prefrontal cortex by Western blotting. The *upper* panel shows data in ethanol-treated (n = 10) and control (n = 8) males and the lower panel data in ethanol-treated (n = 9) and control (n = 8) females. A: Expression of Lipid A in males in PFC. B: Expression of the core element of LPS in males. C: Representative immunoblots of Lipid A and core from the same gel in males. D: Expression of Lipid A in females in PFC. E: Expression of core element in females. F: Representative immunoblots of Lipid A and core from the same gel in females. Western blot data were normalized by β-actin and expressed as a percentage of change over controls. Results represent the mean ± S.E.M. of two technical replicates. No differences were observed between groups. Student's *t* test. LPS, lipopolysaccharide.

In a pilot study, we detected that Lipid A showed expression by Western blot at different molecular weights, which corresponded with receptors and apolipoproteins to which it may bind, as suggested in previous publications (31). Thus, **Fig. 3**A shows the complete profile of Lipid A expression by Western blot when incubated with the antibody against Lipid A. Figure 3A shows a Lipid A positive control (15 μg of *E. coli* LPS (O111:B5)), and the rest of the bands are samples of male control and ethanol-treated animals. The free Lipid A (not bound form) was visualized in a band at approximately 10 kDa. However, the antibody against Lipid A also showed other immunoreactivities at different molecular weights, indicative of the well-known binding of LPS to other molecules, as suggested before (31). Specifically, we detected clear bands at ~31 kDa, ~48 kDa, ~75 kDa, ~96 kDa, and ~210 kDa, which may correspond to the binding of Lipid A to ApoAI, CD14, SR-B1, TLR4, and ApoB, respectively. Immunoblots of each of those proteins incubated with each specific antibody in each case are shown at the right of the panel in Fig. 3A.

To determine whether Lipid A was bound to some of these components, we used two approaches: 1) we checked the expression of each mentioned protein at the specific molecular weight by incubating first with the antibody against Lipid A and then with the antibody of interest, and vice versa; 2) we confirmed by co-IP the binding of the two proteins of interest.

Thus, as an example, Fig. 3B, C represent the binding of Lipid A to TLR4. Figure 3B shows the expression of TLR4 (band at ~96 kDa). The membrane was stripped and then incubated with the antibody against Lipid A, which also detected a band near the same molecular weight. The results of the [LipidA-TLR4]-bound form were normalized to the total expression of TLR4 (free + bound form; incubation with antibody against TLR4) in the PFC (Fig. 3B).

The binding of Lipid A to TLR4 was confirmed twice by co-IP. First, we precipitated the conjugate using an antibody against Lipid A, and then the complex was incubated with the antibody of interest, in this specific case, TLR4 (Fig. 3C, upper panel). To double check this

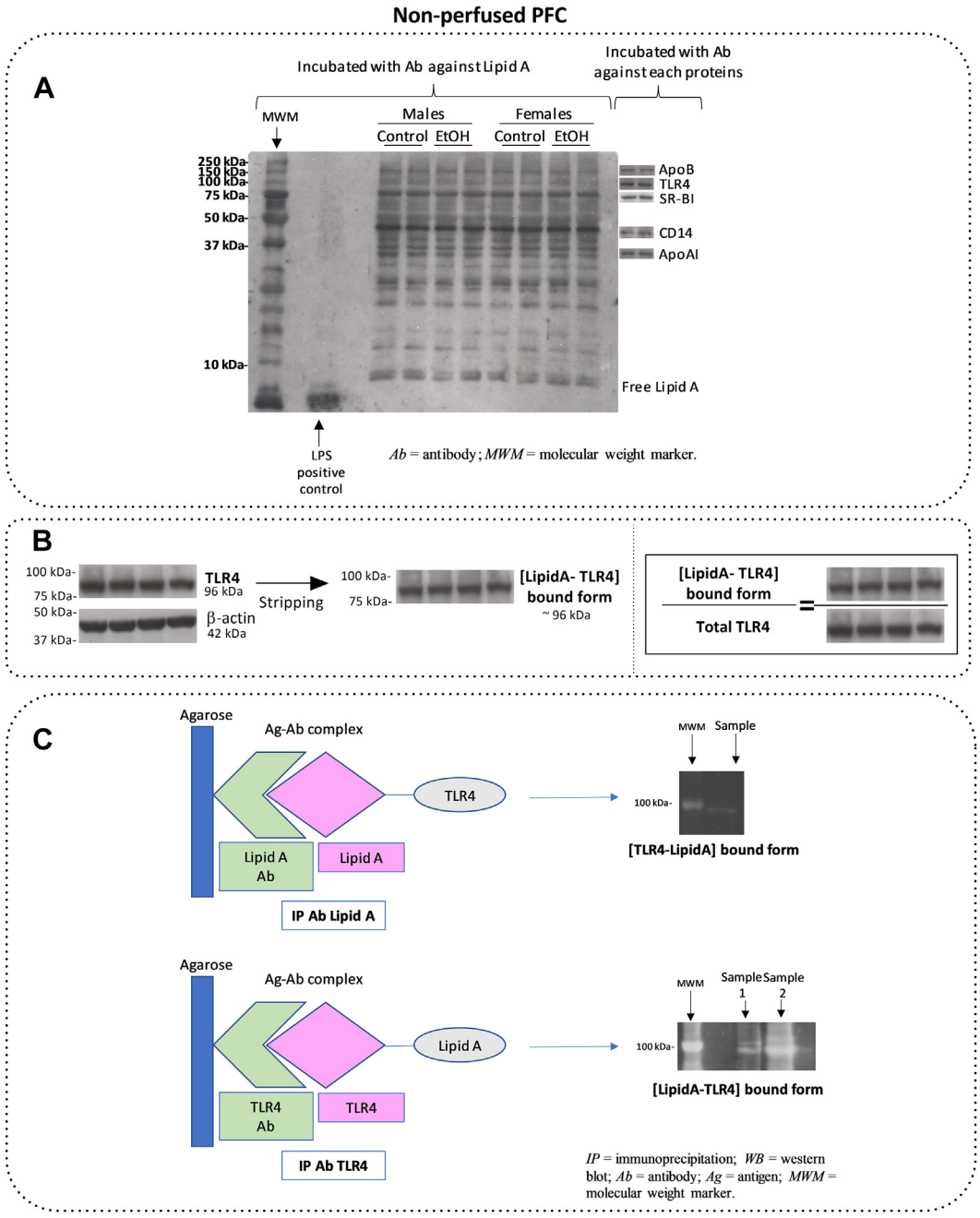


**Fig. 3.** Screening study of Lipid A bound to different molecules in PFC: pilot study with TLR4. A: Representative immunoblot of a membrane from a 18% gel incubated with antibody against Lipid A, where Lipid A showed expression at different molecular weights (left side). On the right side, samples of immunoblots which correspond with receptors and apolipoproteins which Lipid A may bind to. B: Representation of the binding of Lipid A to TLR4. The ratio [LipidA-TLR4]-bound form is an indirect measurement of colocalization of proteins. [LipidA-TLR4]-bound form was detected at 96 kDa and normalized by total TLR4. Blot images in the far-right-side are reused from the left and middle-side in order to represent the normalization (ratio) process. C: Left panel in (C) is a schematic representation of the co-IP process as a direct measure of colocalization of Lipid A and TLR4. Upper panel represents the immunoreactivity obtained from Lipid A immunoprecipitated incubated with an antibody against TLR4. Down panel shows the immunoreactivity obtained from TLR4 immunoprecipitated incubated with an antibody against Lipid A. co-IP, coimmunoprecipitation.

binding, another set of samples was prepared, and the conjugate was precipitated using an antibody against TLR4 first. Then, the complex was visualized by analysis of the immunoreactivity against Lipid A (Fig. 3C, lower panel).

In this pilot study, we showed that our approach of binding of Lipid A and TLR4 by Western blotting was confirmed by co-IP. In a new study, we used both Western blotting and co-IP to check the binding of Lipid A to TLR4 and different apolipoproteins and

their receptors, and the results are presented in the next sections. Co-IP was used as qualitative confirmation of each specific binding of proteins (conjugate first precipitated by Lipid A antibody and then the complex incubated with the antibody of the protein of interest), and we used Western blot analyses to quantify the samples in our study, since the co-IP is limiting tissue technique that needs pooled samples (see Materials and methods section).

In the next sections, we report the studies of the binding of Lipid A with TLR4, ApoAI, ApoB, and ApoE in the different experimental groups.

### TLR4 expression and quantification of LipidA-TLR4-bound form in male and female rats under alcohol or control conditions

We used both Western blotting and co-IP to check the binding of Lipid A to TLR4 and different apolipoproteins. Figure 4A represents the total expression of TLR4 in the PFC of male rats under ethanol or control conditions. We observed that TLR4 was upregulated in ethanol-treated male rats (Fig. 4A; $t_{(16)}$ = 2.484, $P$ = 0.0244), whereas there was no significant effect in females. Notably, at the time-point of tissue extraction (3 h after the last alcohol binge), males

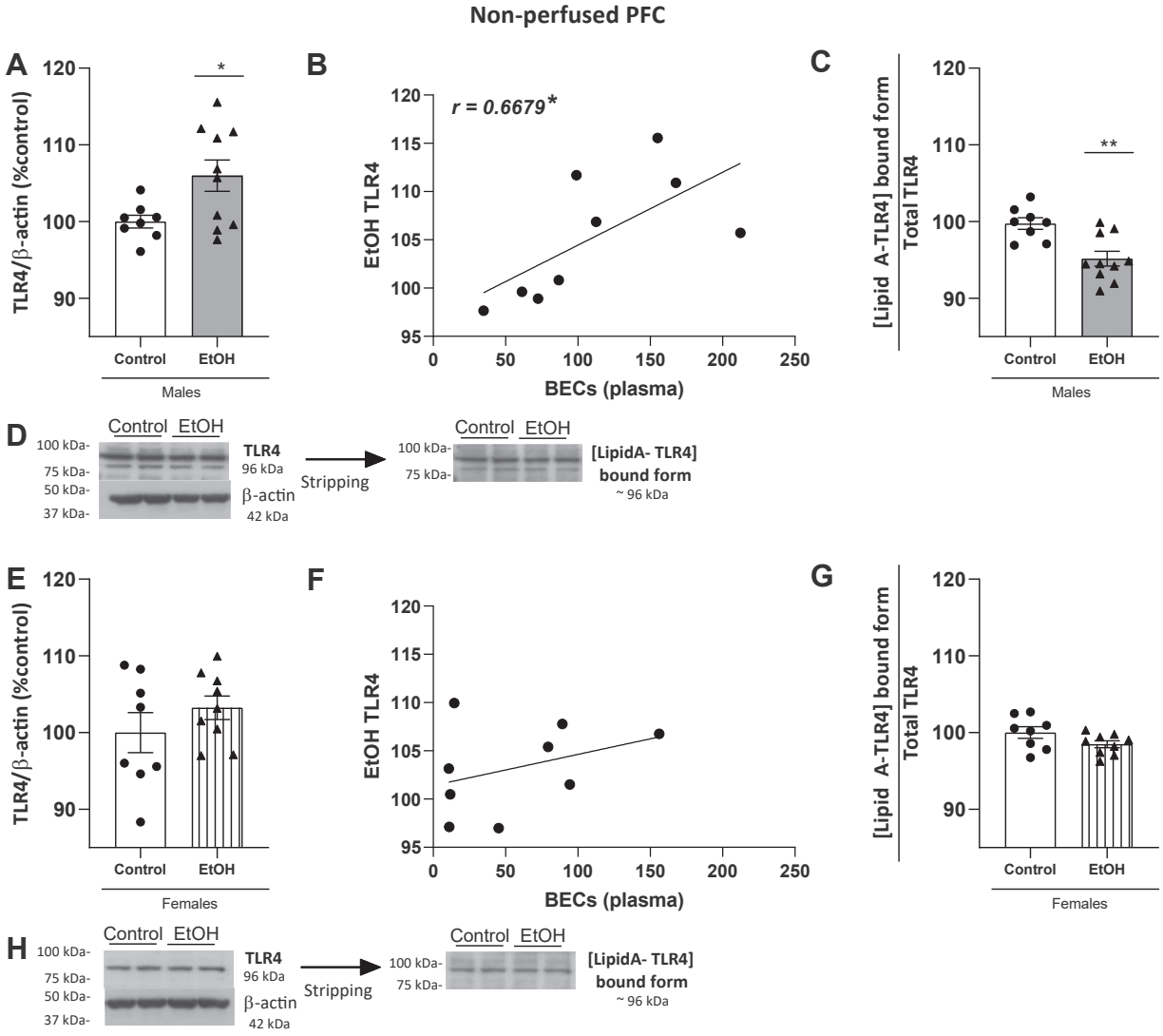


**Fig. 4.** TLR4 and [LipidA-TLR4]-bound form expression by Western blotting and TLR4-BECs linear regression in PFC. The upper panel shows data in ethanol-treated versus control males and the lower panel data in ethanol-treated versus control females. A: TLR4 levels were increased in ethanol-treated males. B: Linear regression between TLR4 in PFC and BECs in plasma in males. The trend line shows the regression analyses for the ethanol-treated group. BECs in plasma were positively correlated with the TLR4 levels in PFC. C: The ratio [LipidA-TLR4]-bound form in males, as indirect measurement of colocalization of proteins, were decreased in ethanol-treated group. Lipid A was detected at 96 kDa and normalized by total TLR4. D: Representative immunoblots of total TLR4 and [LipidA-TLR4]-bound form from the same gel of males. E: Expression of total TLR4 in females. F: Linear regression between TLR4 in PFC and BECs in plasma females. G: The ratio [LipidA-TLR4]-bound form in females. H: Representative immunoblots of total TLR4 and [LipidA-TLR4]-bound form from the same gel in females. Western blot data were normalized by β-actin and expressed as a percentage of change over controls. Results represent the mean ± S.E.M. of two technical replicates. Differences from control group: *$P < 0.05$, **$P < 0.01$ (Student's $t$ test or Pearson's coefficient correlation $r$).

showed higher BECs than females (Table 1), and we found a positive correlation between BECs and TLR4 expression in the PFC in male animals (Fig. 4B, $r = 0.6679$, $P < 0.05$) that was not found in females. The study of binding between TLR4 and Lipid A is represented in Fig. 4C for males. Data are expressed as [LipidA-TLR4]-bound form normalized by the expression of total TLR4. The results indicate that the [LipidA-TLR4]-bound form is decreased in male ethanol-treated animals (Fig. 4C; $t_{(16)} = 3.589$, $P = 0.0025$). Figure 4D shows the representative blots for Western blot analyses in males.

The bottom panel of Fig. 4 shows the same parameters in female animals. As commented before, no significant effect was found in TLR4 expression in female ethanol rats compared to controls in the PFC (Fig. 4E; $t_{(15)} = 1.098$, $P = 0.2897$, n.s.), no correlation of TLR4 with BECs (Fig. 4F, $r = 0.3589$, $P > 0.05$), and no binding of TLR4-Lipid A was found in females (Fig. 4G; $t_{(15)} = 1.769$, $P = 0.0971$, n.s.). Figure 4H shows representative blots in female animals.

### Expression of ApoAI, [LipidA-ApoAI] aggregates, and SR-BI receptors in the PFC in male and female ethanol-treated and control animals

ApoAI was detectable in the PFC of control and ethanol-treated animals by Western blotting (**Fig. 5**). In male rats, there were no differences between the alcohol and control groups in total PFC ApoAI levels (Fig. 5A; $t_{(16)} = 0.2420$, $P = 0.8119$). Regarding the [LipidA-ApoAI]-bound form, we did not observe significant differences between groups (Fig. 5B; $t_{(16)} = 1.209$, $P = 0.2442$). The data showed that SR-BI was detectable in this structure by Western blot, with no changes between the ethanol and control groups (Fig. 5C; $t_{(16)} = 0.02170$, $P = 0.9830$). Figure 5D shows the representative blots for these proteins in males.

In female rats, whereas no differences in total ApoAI levels were detected in the PFC (Fig. 5E, $t_{(15)} = 0.4364$, $P = 0.6687$), we observed an increase in the [Lipid A-ApoAI]-bound form in the ethanol group compared to controls (Fig. 5F; Mann–Whitney, $U = 0$, $P < 0.001$). Expression of the ApoAI receptor SR-BI was not altered in females (Fig. 5G; $t_{(15)} = 0.6976$, $P = 0.4961$). Blots are represented in Fig. 5H for female animals.

Figure 5I shows a representative diagram of the precipitated complex in the co-IP procedure using pooled samples (left panel) and a representative image of the confirmation of the binding between Lipid A and ApoAI by co-IP in the PFC (right panel).

### Expression of ApoB, [Lipid A-ApoB] aggregates, and LDLr in the PFC of male and female ethanol-treated and control animals

ApoB levels were detectable in the PFC in all experimental groups, and quantifiable analyses are shown in **Fig. 6**. There were no significant differences in total ApoB levels between the alcohol and control groups in males (Fig. 6A; $t_{(15)} = 1.381$, $P = 0.1876$). However, we observed a clear increase in the [LipidA-ApoB]-bound form in ethanol-treated male animals versus controls (Fig. 6B; $t_{(16)} = 2.448$, $P = 0.0263$), and interestingly, levels of the ApoB receptor LDLr in the PFC were also upregulated in the ethanol group in males (Fig. 6C; $t_{(16)} = 2.253$, $P = 0.0387$). Figure 6D shows representative blots for these proteins in male rats.

In females, there were no significant changes between groups in total ApoB levels (Fig. 6E; $t_{(15)} = 0.09525$, $P = 0.9254$), the [LipidA-ApoB]-bound form (Fig. 6F; $t_{(15)} = 0.9816$, $P = 0.3419$), or the expression of LDLr (Fig. 6G; $t_{(15)} = 1.235$, $P = 0.2360$). Figure 6H shows the representative blots for these proteins in females.

The binding of Lipid A with ApoB was confirmed by visualization of the immunoprecipitated complex by co-IP in pooled samples (Fig. 6I).

### Expression of ApoE, [Lipid A-ApoE] aggregates, and ApoER2 in the PFC of male and female ethanol-treated and control animals

Although ApoE levels in plasma were detected by ELISA under our experimental conditions (see Results Plasma LPS, LBP, and Apos (AI, B, and E) levels in male and female ethanol-intoxicated and control animals), they were clearly detected in the PFC in both the control and ethanol groups (**Fig. 7**), as expected due to its astrocyte origin.

Figure 7 shows that there were no differences between the alcohol and control groups in total ApoE levels, in the [LipidA-ApoE]-bound form (Fig. 7A, B; $t_{(16)} = 0.01856$, $P = 0.29854$; $t_{(16)} = 0.1418$, $P = 0.8890$, respectively) or in ApoER2 expression (Fig. 7C; $t_{(16)} = 1.082$, $P = 0.2953$) in the PFC of males. Blots are represented in Fig. 7D.

Similarly, no differences were found in the total form of ApoE, the [LipidA-ApoE]-bound form, and ApoER2 expression in females (Fig. 7E–G; $t_{(15)} = 1.553$, $P = 0.1413$; Mann–Whitney $U = 34$; $P > 0.05$; $t_{(15)} = 0.2900$, $P = 0.7758$ n.s., respectively). Representative blots in Fig. 7H.

### Detection of LPS components (Lipid A and core), TLR4, and Apos in the cerebellum of male and female ethanol-treated and control animals

The whole study was repeated in the cerebellum (nonperfused animals), both in the vermis and in the cerebellar hemispheres (Hcb) (Supplemental Results 2.2). As mentioned before, Lipid A, Core, and TLR4 were also detectable within the cerebellum of male and female animals, with no differences between experimental groups (Supplemental Results 2.2, Supplemental Fig. S3).

The expression of ApoAI and ApoB was also checked in the cerebellum, together with their receptors and their bound forms to Lipid A (Supplemental Results 2.2). No significant differences were found between experimental groups (Supplemental Figs. S4 and S5 for studies with ApoAI and ApoB, respectively).

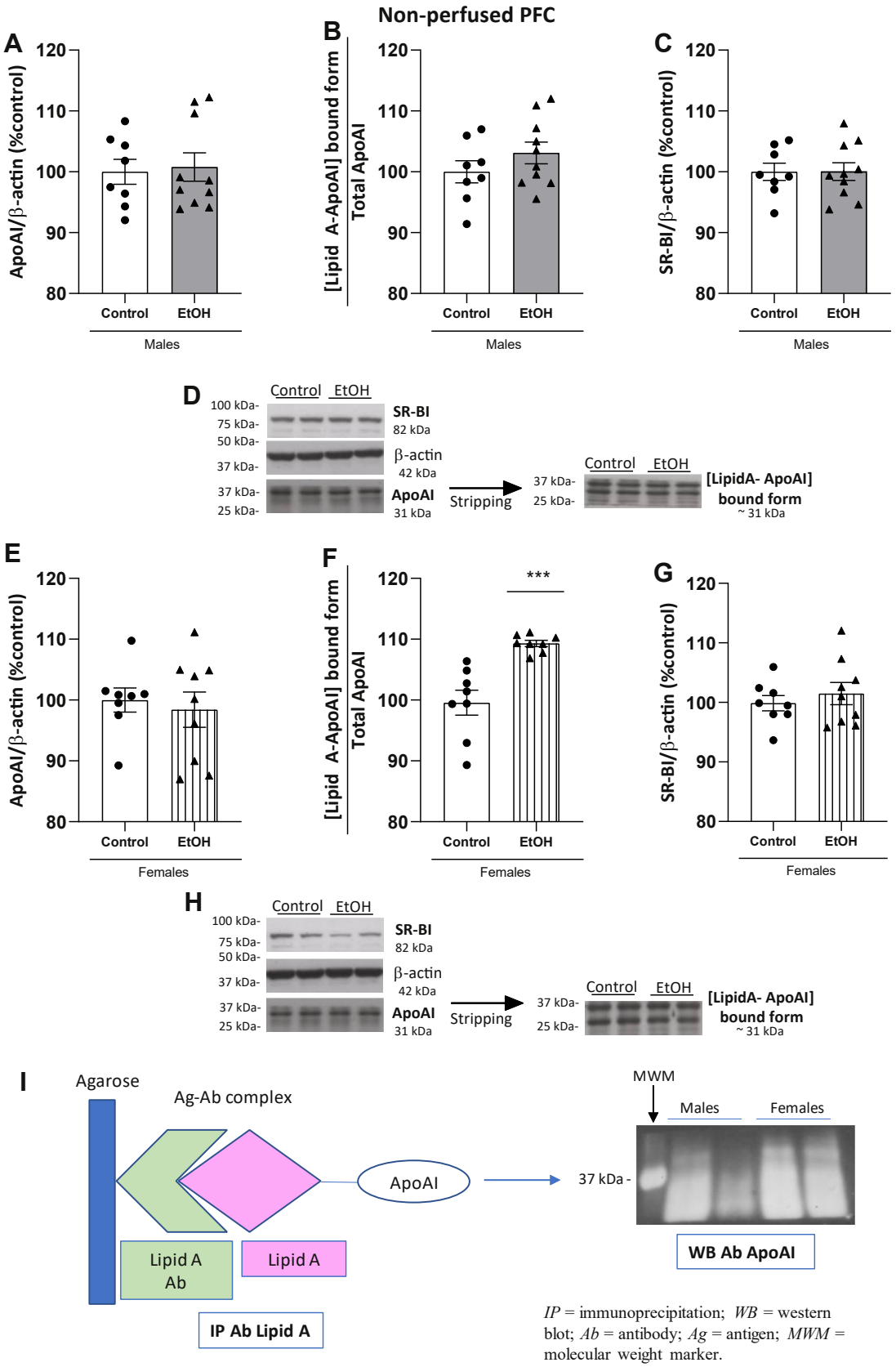


**Fig. 5.** Expression of ApoAI and [LipidA-ApoAI] by Western blotting and its receptor SR-BI in PFC. The upper panel shows data in ethanol-treated (n = 10) and control (n = 8) males and the lower panel data in ethanol-treated (n = 9) and control (n = 8) females. A: Expression of total ApoAI in males. B: The ratio [LipidA-ApoAI]-bound form in males, as indirect measurement of colocalization of proteins (Lipid A detected at 31 kDa and normalized by total ApoAI). C: Expression of SR-BI in males. D: Representative immunoblots of total ApoAI, [LipidA-ApoAI]-bound form, and SR-BI in males from the same gel. E: Expression of total ApoAI levels in

The study with ApoE is represented in Supplemental Fig. S6 (Supplemental Results 2.2) and showed sex differences in the expression of ApoER2, with upregulation in ethanol-treated males versus controls (Supplemental Fig. S6C; Mann–Whitney U = 10, $P < 0.05$) and downregulation in ethanol-treated females (Supplemental Fig. S6G; $t_{(14)} = 3.133$, $P = 0.0073$).

### Analyzing the contribution of cerebral blood flow to the presence of bacterial products within the brain

This study investigated the presence of bacterial components in their free form or bound to apolipoproteins within brain areas affected by alcohol, such as the PFC and cerebellum. Although it was not a fundamental objective of the present study, we performed a pilot experiment to ascertain the influence of blood flow within the brain in the changes observed. In this pilot study, we compared perfused (blood removed) and nonperfused female control rats regarding the presence of free Lipid A or its aggregates with ApoAI or TLR4.

**Figure 8**A shows that free Lipid A levels were lower when the blood was removed from the animals compared with nonperfused rats ($t_{(13)} = 4.020$; $P = 0.0015$), according to the well-known peripheral origin of LPS. However, levels of free Lipid A were still detectable in perfused animals (Fig. 8A). Interestingly, we did not observe differences in the LipidA-TLR4 aggregates (Fig. 8C, D; Mann–Whitney, U = 24, $P > 0.05$; $t_{(14)} = 1.022$; $P = 0.3239$) or LipidA-ApoAI (Fig. 8E,F; $t_{(14)} = 0.2082$; $P = 0.8381$; $t_{(14)} = 0.7757$; $P = 0.4508$) in the PFC of perfused versus nonperfused animals. These results suggest that an important percentage of Lipid A is retained in the blood vessels within the PFC but the aggregated forms with receptors or apolipoproteins are present within the cerebral blood vessel structures or even in the brain parenchyma, as suggested before by immunohistochemistry procedures (31).

## DISCUSSION

This groundbreaking study reports the presence of parts of LPS bound to apolipoproteins in the brain of animals exposed to alcohol binge intoxications, being the apolipoprotein involved in the aggregate different in male and female rats. Specifically, we detected the presence of Lipid A, the endotoxic component of LPS, bound to ApoAI in the PFC of female rats exposed to alcohol, whereas Lipid A was found bound to ApoB in male ethanol-treated animals, which also showed increases in LDLr (ApoB/LDL main receptor) in the PFC. ApoAI was also clearly upregulated in the plasma of female ethanol-treated animals versus controls, an effect that was not observed in males. Interestingly, males in the ethanol group showed an upregulation of TLR4, a signature of neuroinflammation, in the PFC versus controls, which was not observed in females, and a reduced binding of TLR4 to Lipid A. These alterations were not observed within the cerebellum. These results suggest the existence of a differential apolipoprotein-mediated mechanism to bind LPS components in the brain of male and female animals which underwent alcohol binge intoxications. Implications for alcohol-induced neuroinflammation in the PFC are discussed below.

It is well known that alcohol intoxications induce an increase in plasma LPS due to leaky gut and liver clearance downregulation (7, 11, 15, 39–41). In the present study, despite a tendency in an overall alcohol effect, the LPS increase did not reach statistical significance, which was surprising since we previously reported LPS increases under the same binge procedure (10, 11, 15), and it is probably attributed to the slightly younger age of these animals compared with the mentioned studies. We observed, nonetheless, clear significant elevations in LBP both in females and males in response to ethanol binges, which has also been considered a marker of bacterial translocation and inflammation (42). In any case, several components of the LPS molecule, such as Lipid A and Core, were detected in the PFC and cerebellum of both sexes in the current study, with no significant differences between the control and ethanol groups. We performed a pilot study demonstrating that in perfused control animals (blood removed), the free Lipid A was lower than in intact (nonperfused) brains. These results suggest that a percentage of the Lipid A observed in this study is located outside of the BBB and other percentage may infiltrate it. This result is in agreement with mechanistic studies perfusing I-LPS where it was found that half of the I-LPS associated with brain permeated the BBB entering the parenchyma space and about half was sequestered by the capillary bed into endothelial cells (30). The Lipid A could be located at the luminal (blood-facing) or abluminal (brain-facing) side of the brain endothelial cells, but this information was not an objective of the present study. It is known that LPS may bind receptors located in brain endothelial cells, including TLR4, inducing the release of proinflammatory cytokines, and this release can

females. F: The ratio [LipidA-ApoAI]-bound form in female, as indirect measurement of colocalization of proteins, was increased in ethanol-treated females. Lipid A was detected at 31 kDa and normalized by total ApoAI. G: Expression of SR-BI levels in female rats. H: Representative immunoblots of total ApoAI, [LipidA-ApoAI]-bound form, and SR-BI in females from the same gel. I: Colocalization of Lipid A and ApoAI by co-immunoprecipitation (co-IP). Left panel in (I) is a schematic representation of the co-IP process. Co-IP is a direct measure of colocalization of Lipid A and ApoAI. Right panel shows a representative image of the co-IP. Results were obtained by pool of samples in the same experimental group and are descriptive. Quantification of data was done by Western blotting, and it is shown in (B) and (F). Results represent the mean ± S.E.M. of two technical replicates. Differences from control group: ***$P < 0.001$ (Student's $t$ test or Mann-Whitney (3F)). SR-BI, scavenger receptor class B type 1.

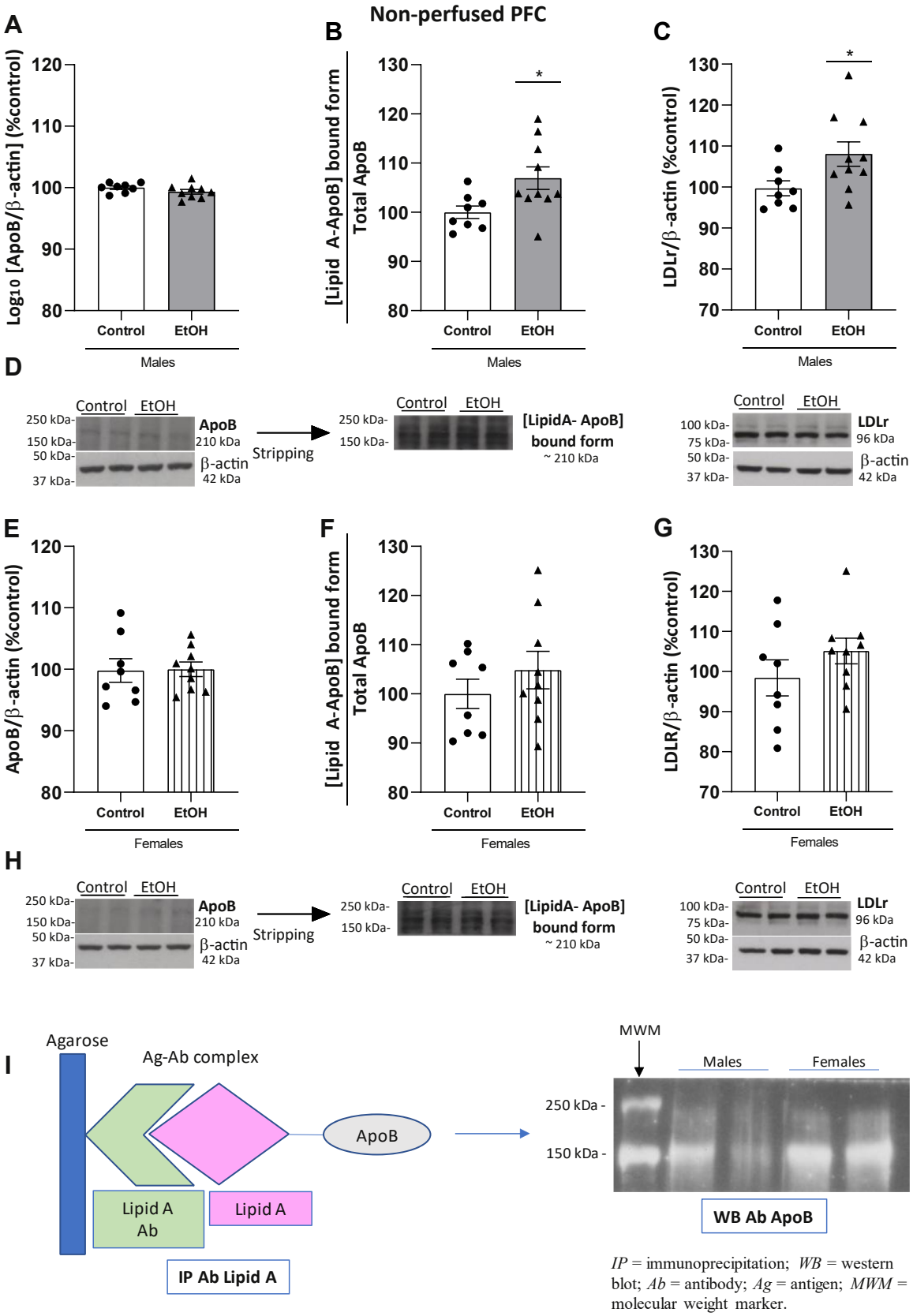


**Fig. 6.** Expression of ApoB and [LipidA-ApoB] by Western blotting and its receptor LDLr in PFC. The upper panel shows data in ethanol-treated (n = 10) and control (n = 8) males and the lower panel data in ethanol-treated (n = 9) and control (n = 8) females. A: Expression of total ApoB in males. B: The ratio [LipidA-ApoB]-bound form in males, as indirect measurement of colocalization of proteins, was increased in ethanol-treated group. Lipid A was detected at 210 kDa and normalized by total ApoB. C: LDLr levels were increased in ethanol group in males. D: Representative immunoblots of total ApoB, [LipidA-ApoB]-bound form, and LDLr in males

occur at either side of the brain-endothelial cells (43). However, we observed that the aggregates were maintained in perfused animals at similar levels than in nonperfused rats, suggesting that LipidA-apolipoprotein bound forms could infiltrate the brain, as we reported in a previous immunohistochemical study (31). Interestingly, in the ethanol groups, Lipid A was bound to different apolipoproteins to a greater extent than in control animals and the apolipoprotein to which Lipid A binds differs in male and female animals. These results may open new avenues to the understanding of the crosstalk among alcohol, LPS, and neuroinflammation.

ApoAI is mainly synthesized in the liver and intestine (44) and works to remove excess cholesterol from cells and send LPS to the liver for its elimination (45, 46). In our study, we did not observe basal differences in the amount of ApoAI in plasma between male and female control animals, although it has been reported that women appear to have higher plasma HDL-ApoAI levels under physiological conditions than men (47). However, we found an increase in plasma ApoAI levels in the female ethanol group versus female controls, despite no changes in plasma HDL were found. This is in agreement with recent studies indicating that, rather than changes in HDL plasma levels, what varies the HDL functionality under pathological conditions is the HDL-protein cargo (i.e., ApoAI) (48). Plasma LDL, which transports mainly ApoB, plays an important role in lipid transport (45, 49), and it appears to be higher in men than in women (47). In our study, male animals also had higher plasma LDL levels than females, disregarding of the experimental treatment. In humans, moderate alcohol consumption has been associated with increases in plasma HDL levels (50) in a dose-dependent fashion and turnover of ApoAI (51), although recent studies suggest that long-term alcohol consumption may decrease HDL serum levels in women (52). Reports about the effect of binge drinking on plasma lipoproteins are very scarce in the field, with some reporting increases in plasma HDL and decreases in LDL profiles in heavy drinkers (53). Our results highlight one of the sex differences found in this study, since ApoAI plasma levels were not increased in males under ethanol intoxications. The higher levels of plasma ApoAI found in female animals of the ethanol group could indicate that females activate an ApoAI-mediated protective mechanism to neutralize LPS, since it is known that HDL, which integrates mainly ApoAI in the periphery, binds LPS and helps in its transport to the liver for elimination (46, 54).

ApoAI is also naturally present in the brain since it is known that it crosses the BBB back and forth from the circulation to the brain (55, 56), and it has been found in the CSF (57–59). In this study, we detected ApoAI in the PFC and cerebellum of both male and female rats and observed that Lipid A was specifically bound to ApoAI in the PFC of females exposed to alcohol versus controls. This effect was not observed in male animals. The upregulation of the LipidA–ApoAI complex in the PFC is parallel with an absence of TLR4 overexpression in females exposed to ethanol, which could be interpreted in different ways, as will be discussed below. As mentioned before, the wash out of the vascular space did not change the expression of LipidA-apolipoprotein aggregates within the brain in our pilot study, suggesting that they may infiltrate the brain. Similar aggregates were previously found in the blood-brain interfaces (ie tanycyte-like cells, ependymal cells, and brain-endothelial cells) but also within the cerebral parenchyma, such in astrocytes in the medulla oblongata (31). It is very possible that the Lipid A-apolipoprotein aggregates are part of the BBB (ie endothelial cells) and have a role in the vascular homeostasis, affecting proinflammatory signals at one or the other side of the barrier (28, 43). Thus, there are several possibilities to explain the Lipid A-ApoAI binding in the PFC: 1) the elevated levels of peripheral ApoAI found in the females of the ethanol group would bind Lipid A in the plasma, and the complex is transported to the blood-brain interfaces or brain parenchyma within the PFC; 2) the aggregate (Lipid A and ApoAI joined form) takes place within the BBB structures (ie endothelial cells) in the PFC at the luminal (blood-facing) or abluminal (brain-facing) side of it. Alternatively, both possibilities could be happening at the same time, and, in any case, these results would suggest that LPS components may signal in the PFC, but not in the cerebellum, after alcohol binge intoxications helped by a mechanism dependent on apolipoproteins. These hypotheses need further confirmation in future studies, as well as the precise mechanisms and the functional consequences of the binding. In any case, SR-BI appears to be responsible for HDL internalization and transcytosis across the BBB (60), but its participation in the processes described here is uncertain, since no changes in the

from different gels. E: Expression of total ApoB levels in females. F: The ratio [LipidA-ApoB]-bound form in female, as indirect measurement of colocalization of proteins (Lipid A was detected at 210 kDa and normalized by total ApoB). G: Expression of LDLr levels in female rats. H: Representative immunoblots of total ApoB, [LipidA-ApoB]-bound form, and LDLr in females from different gels. I: Colocalization of Lipid A and ApoB by co-IP. Left panel in (I) is a schematic representation of the co-IP process. Co-IP is a direct measure of colocalization of Lipid A and ApoB. Right panel shows a representative image of the co-IP. Results were obtained by pool of samples in the same experimental group and are descriptive. Quantification of data was done by Western blotting, and it is shown in (B) and (F). Results represent the mean ± S.E.M. of two technical replicates. Differences from control group: $^{*}P < 0.05$ (Student's *t* test). co-IP, coimmunoprecipitation; LDL, low-density lipoprotein; LDLr, LDL receptor.

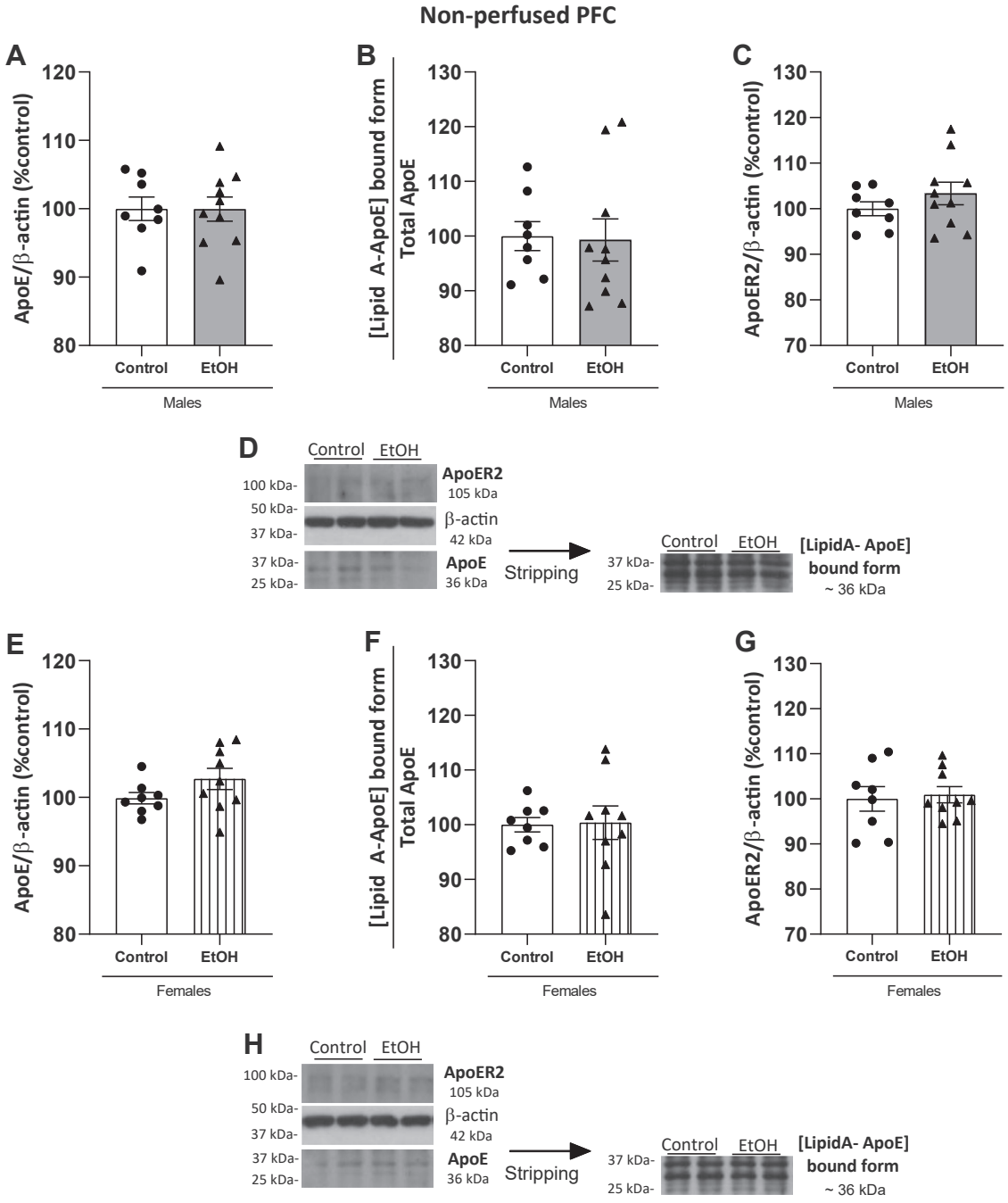


**Fig. 7.** Expression of ApoE and [LipidA-ApoE] by Western blotting and its receptor ApoER2 in PFC. The upper panel shows data in ethanol-treated (n = 10) and control (n = 8) males and the lower panel data in ethanol-treated (n = 9) and control (n = 8) females. A: Expression of total ApoE in males. B: The ratio [LipidA-ApoE]-bound form in males, as indirect measurement of colocalization of proteins (Lipid A was detected at 36 kDa and normalized by total ApoE). C: ApoER2 levels in males. D: Representative immunoblots from the same gel of total ApoE, [LipidA-ApoE]-bound form, and ApoER2 in males. E: Expression of total ApoE levels in females. F: The ratio [LipidA-ApoE]-bound form in female, as indirect measurement of colocalization of proteins (Lipid A was detected at 36 kDa and normalized by total ApoE). G: Expression of ApoER2 levels in female rats. H: Representative immunoblots from the same gel of total ApoE, [LipidA-ApoE]-bound form, and ApoER2 in females. Western blot data were normalized by β-actin and expressed as a percentage of change over controls. Results represent the mean ± S.E.M. of two technical replicates. There were no differences between groups (Student's *t* test, $P > 0.05$). ApoER2, ApoE receptor 2.

expression of SR-BI were observed in any condition in this study.

The sequestration of Lipid A by ApoAI in the female brain under ethanol intoxications could be interpreted as a mechanism to protect it against neuroinflammation, preventing a LipidA-mediated activation of TLR4 signaling. In this line and as mentioned before, LipidA-ApoAI binding in the PFC was increased in alcohol-administered female rats, where no TLR4 signature (sign of neuroinflammation)

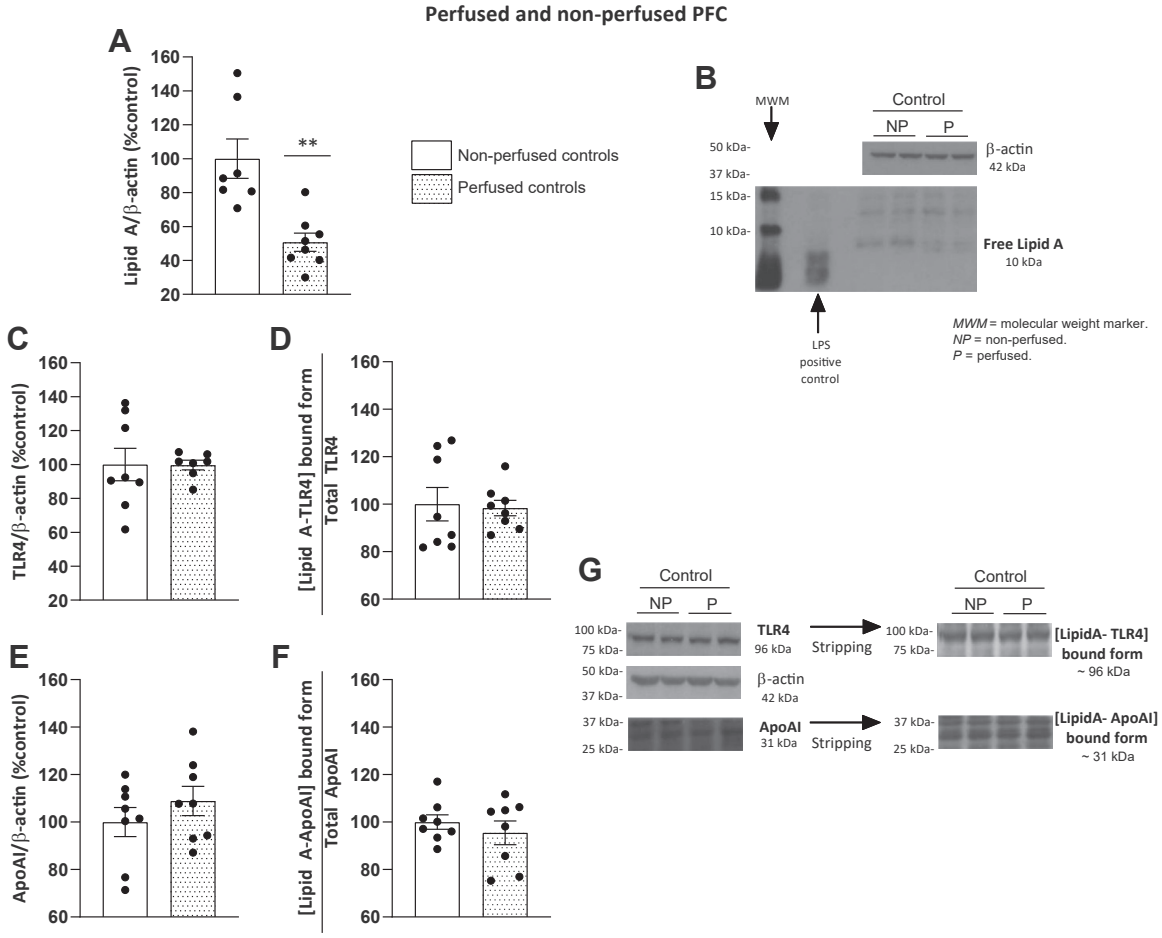


**Fig. 8.** Presence of free Lipid A and [LipidA-protein] aggregates in the PFC of perfused and nonperfused female controls. A: Expression of free Lipid A. B: Representative immunoblots of Lipid A. C: TLR4 levels. D: Ratio [LipidA-TLR4]-bound form. Lipid A was detected at 96 kDa and normalized by total TLR4. E: ApoAI levels. F: Ratio [LipidA-ApoAI]-bound form Lipid A was detected at 31 kDa and normalized by total ApoAI. G: Representative immunoblots of total TLR4, total ApoAI, [LipidA-TLR4], and [LipidA-ApoAI]-bound forms from the same gel. Western blot data were normalized by β-actin and expressed as a percentage of change over nonperfused controls. Results represent the mean ± S.E.M. of two technical replicates. Differences between groups: $^{**}P < 0.01$ (Student's *t* test).

was found. However, in males, alcohol intoxications induced a clear upregulation of TLR4 in the PFC, which was also observed in previous studies (13, 15, 61), and no binding of Lipid A with ApoAI was found. The anti-inflammatory actions of ApoAI have been described in several conditions, such as sepsis (62), and decreases in plasma ApoAI have been associated with the severity of Alzheimer's disease (48). Clearly, further research is needed to ascertain a possible protective role of ApoAI in the female brain under alcohol conditions. Intriguingly, in a very recent study, Radford-Smith and colleagues showed that intraperitoneal administration of HDL together with LPS in mice shuttles the endotoxin LPS to the brain and promotes neuroinflammation, whereas the co-administration of LDL with LPS has anti-neuroinflammatory properties (63). The binding of Lipid A with ApoAI could then be interpreted as a protective strategy to capture LPS, in line with most of the studies, or, counterintuitively, as a strategy to help LPS access the brain, as suggested elsewhere (63). Indeed, it has been suggested that lipoproteins may act with a double function: they may capture and clear LPS from blood and tissues, but they may induce inflammatory responses elsewhere (63). The dance between LPS and the lipoprotein subclasses is very complex since, although LPS displays greater affinity for HDL, it can be transferred from HDL (ApoAI) to LDL (ApoB) in response to an acute-phase response to infection (27). This is in accordance with our study, since we observed that in alcohol-treated animals, Lipid A is bound to ApoAI in the PFC of females, whereas it is bound to ApoB in males, who showed a clearer neuroinflammatory response in the PFC. Interestingly, one of the molecules in charge of this transfer of LPS from HDL to LDL is plasma LBP (27), which was found to be increased in all ethanol-treated animals versus controls. LBP is considered an even stronger neutralizing molecule against LPS-induced inflammation than ApoAI (64), suggesting that the female ethanol-treated rats in this study were

double protected by elevations in both ApoAI and LBP plasma levels.

Unlike ApoAI, it is believed that ApoB cannot cross the BBB (56), and it is mainly synthesized in the liver. However, some studies have confirmed the presence of ApoB in brain endothelial cells in mice (65), suggesting that ApoB could cross the BBB indeed. Other studies have shown that brain ApoB bound to αβ plaques in transgenic Alzheimer's disease mice (66, 67). Interestingly, LDLr is expressed in neurons and glial cells (45) and may facilitate ApoB passage from blood to brain by transcytosis (68). Here, we detected an upregulation of LDLr expression in the PFC of males that underwent alcohol administrations, and these animals also showed an increase in the LipidA-ApoB-bound form compared to controls. Altogether, these data indicates that the Lipid A bound a specific apolipoprotein in males and females under alcohol intoxications, and this result deserves further investigation. It is possible that differences in the magnitude of the acute-phase response induced by alcohol in males and females in our study account for this effect, since LPS may be exchanged between lipoprotein subclasses according to the neuroimmune capacity, as discussed above.

We also checked the presence of ApoE, one of the major apolipoproteins in the CNS, which is highly expressed by astrocytes but also by oligodendrocytes and microglia (69). ApoE is involved in the transport of cholesterol and other lipids through the bloodstream and the CNS (57, 59), and the ApoE4 isoform has been widely studied for its participation in neuroinflammation and cognitive decline (70) in several neurological disorders (71, 72), including alcohol abuse (73, 74). In our study, there were no differences in the expression of ApoE between experimental groups or its binding to Lipid A in the PFC, both in males and females. We did not distinguish between the ApoE isoforms, which is probably a limitation of the study. The levels of ApoE4 in plasma were under the limit of detection in our experiment. It is believed that brain ApoE does not cross to the periphery, but it may cross the BBB when it is bound to HDL (48). Indeed, the source of peripheral ApoE appears to be mainly the liver, with the brain and endocrine cells contributing little if any to plasma ApoE levels (48). In our experiment, we did not study the levels of HDL within the brain, as HDL in the periphery is mainly enriched with ApoAI, and its synthesis in glial cells is enriched with ApoE (48). In any case, ApoE appears not to play a fundamental role in the hypothesis of our study, although given the importance that the isoform ApoE4 plays in the context of alcohol and neuroinflammation, further studies are needed to ascertain possible implications of this specific isoform of ApoE.

This study highlights several events in the brain of male and female animals exposed to alcohol binge episodes. First, the specific binding of Lipid A to different apolipoproteins in the brains of males and females exposed to alcohol. Second, we detected different BECs achieved by males and females with the same doses of alcohol, with lower levels observed in females at the time of blood extraction. It is possible that the metabolism of alcohol is faster in females, as suggested elsewhere (75–78), and the peak of BECs occurred at early times. Indeed, other markers are indicative of the activation of the acute-phase response in females, such as the plasma corticosterone levels, which was higher in females of the ethanol group. This higher corticosterone response in females than males after alcohol exposure was expected, according to the literature (79–81). Finally, as mentioned before, we found here a TLR4 upregulation in the PFC of males that underwent alcohol gavage administrations, which is in accordance with previous literature about the neuroinflammatory actions of ethanol in this area (13, 15, 61). In this study, the effect of ethanol on TLR4 upregulation in the PFC was not significant in females. Notably, disparities in the effects of alcohol in females versus males have been noted. Whereas some authors suggest that females may be more vulnerable to alcohol toxic effects than males (82, 83), other authors have shown that female rat brains appear to be more resistant to oxidative damage (84). Comparative studies showing the influence of ethanol in males and females using the same experimental approach at a time are still scarce. Biological differences between males and females and hormonal factors could interfere with the responses observed. For example, sex hormones in females may contribute to a possible neuroprotector effect, as has been suggested elsewhere (85, 86). In this study, the reproductive cycle was monitored in the females during experimentation, and no synchronization was observed among them. The lack of significance in TLR4 expression in alcohol-administered female rats in this study may reflect the protective effect of ApoAI in females, although we cannot discard that the peak of neuroinflammation in females was in an increasing/decreasing phase at the time-point when the sample was obtained (three hours after the last ethanol administration).

We are aware of the limitations of the study, some of which have also been described above: 1) Here, we reported several sexual differences regarding the expression of apolipoproteins, lipoproteins, BELs, corticosterone, etc. in plasma, but data from Western blot were uploaded and analyzed independently for each sex, which limits the total comparison between male and females in both alcohol and control conditions. In spite of this limitation of the study, we were able to identify a differential affinity for apolipoproteins to bind LPS components in male and female ethanol-treated animals, which constitutes an important result of this study. Altogether, these are undoubtedly novel results that open up doors in the field, but further studies are necessary to report results from a complete sexual difference perspective (ie

differences in the expression levels in control animals, etc). 2) The choice of measuring Lipid A, among other parts of LPS, was based on its ability to interact with lipoproteins such as HDL through its Lipid A backbone (54). Studying how other parts of LPS, such as the Core, may interact with apolipoproteins is a goal for future studies. Additionally, in this study, we did not discern whether the binding of Lipid A to apolipoproteins takes place in the periphery, allowing the transport of LPS to the brain, or whether it takes place within the brain or in both compartments. It has been documented that LPS can alter BBB tight junction proteins and cross into the brain (87), although other authors have reported minimal penetration into the brain (30). 3) With this methodology, we cannot assure that the LPS components were able to cross the BBB. The pilot study showing decreases in LipidA in perfused animals but not in the aggregates suggest that the LipidA-apolipoprotein bound forms infiltrated in the brain, as demonstrated in our previous study with immunohistochemistry (31), but additionally, studies will be required to completely demonstrate it. Other studies have shown partial permeability of LPS to the brain parenchyma in different conditions and according to the dose (30) and by action of HDL or apolipoproteins (63, 88). Additionally, here we did not focus on the cellular specific localization of the aggregates and the response to this question needs of future and complementary immunohistochemical studies.

## CONCLUSION

In summary, our study shows the presence of small LPS components (Lipid A) bound to ApoAI in females and to ApoB in males in the PFC of animals exposed to alcohol intoxications, and no effects were found within the cerebellum. The impact of alcohol binges on TLR4 upregulation was observed in the PFC of alcohol-treated males, despite plasma LPS-binding protein was elevated in both sexes. Sexual differences were found in plasma ApoAI and ApoB expression, LDL, BECs, and corticosterone levels. The specific mechanism of the binding of LPS to different apolipoproteins, the cellular localization of the aggregates, and its functional consequences under alcohol intoxication conditions deserve further investigation to better understand the crosstalk between alcohol, neuroinflammation, and the neuroimmune response, as well as an exhaustive analysis from a sex/gender perspective.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Welfare Committee of Complutense University of Madrid (reference: PROEX 312/19) following European legislation (2010/63/EU).

## Data availability

All raw data generated in this study (such as complete Western blot images) are clearly classified, stored, and available to reasonable request to the corresponding author (lorio@psi.ucm.es).

*Supplemental data*

This article contains supplemental data.

*Author contributions*

G.-B. B. and O. L. conceptualization; L.-V. L. and O. L. methodology; L.-V. L., M. M., and E. B. investigation; L.-V. L. and O. L. formal analysis; L.-V. L. writing–original draft; G.-B. B. and O. L. writing–review and editing; O. L. project administration; O. L. funding acquisition.

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*Funding and additional information*

This work was supported by FEDER (European Union)/ Ministerio de Ciencia, Innovación y Universidades (MICINN), Agencia Estatal de Investigación (AEI), Spain (to O. L.) (grant numbers RTI2018-099535-B-I00 and PID2021-127256OB-I00). E. B. and L.-V. L. are recipients of predoctoral scholarships FPU18/01575 and PRE2019-087771, respectively. O. L. is a member of the Instituto de Salud Carlos III RICORS – RIAPAd, Grant RD21/0009/0027 (European Union - NextGenerationEU, Fondo Europeo de Desarrollo Regional FEDER; Mecanismo para la Recuperación, Transformación y Resiliencia [PRTR]).

*Conflict of interest*

The authors have no conflicts of interest to declare.

*Abbreviations*

ABD, alcohol binge drinking; ApoER2, ApoE receptor 2; BBB, blood brain barrier; BEC, blood ethanol concentration; CD14, cluster of differentiation 14; CNS, central nervous system; HDL, high-density lipoprotein; LBP, LPS-binding protein; LDL, low-density lipoprotein; LDLr, LDL receptor; LPS, lipopolysaccharide; PFC, prefrontal cortex; SR-BI, scavenger receptor class B type 1; VLDL, very low-density lipoprotein.

Manuscript received August 9, 2023, and in revised form January 5, 2024. Published, JLR Papers in Press, January 29, 2024, https://doi.org/10.1016/j.jlr.2024.100509

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