UNIVERSITAT DE BARCELONA

Mouse model of fetal alcohol spectrum disorders according to two human alcohol drinking patterns and the role of epigallocatechin gallate in their prevention. Epigallocatechin gallate bioavailability study in humans

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Mouse model of fetal alcohol spectrum disorders according to two human alcohol drinking patterns and the role of epigallocatechin gallate in their prevention. Epigallocatechin gallate bioavailability study in humans.

Thesis submitted by Laura Almeida Toledano in partial fulfilment of the requirements for the degree of Doctor of Philosophy from University of Barcelona

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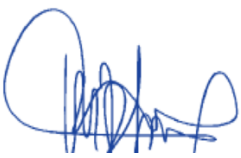
The thesis entitled *"Mouse model of fetal alcohol spectrum disorders according to two human alcohol drinking patterns and the role of epigallocatechin gallate in their prevention. Epigallocatechin gallate bioavailability study in humans"* is submitted by Laura Almeida Toledano for the PhD degree of Doctor of Medicine and Translational Research of the University of Barcelona under the direction of María Dolores Gómez Roig, Professor of Obstetrics and Gynecology at Barcelona University, and Vicente Andreu Fernández, senior postdoctoral researcher at August Pi i Sunyer Biomedical Research Institute (*IDIPAPS*).

The co-directors declare that Laura Almeida Toledano has conducted the research work presented in this thesis at the Barcelona Center for Maternal-Fetal and Neonatal Medicine (*BCNatal*) (Sant Joan de Déu Barcelona Hospital), the Sant Joan de Déu Research Institute (*IRSJD*), and the Childhood and Environment Research Group (*GRIE*) in *IDIBAPS*, under their supervision. Laura Almeida Toledano has substantially contributed to all the studies here presented. She was involved in the design of the studies, carried out experimental work, managed the databases, analyzed the results, wrote the manuscripts, and reviewed the final version of the articles submitted for publication and included in this thesis. The co-directors state that none of the co-authors has used or is going to use any of the articles here presented in future PhD thesis.

Barcelona, April 2021



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Real knowledge is to know
the extent of one's ignorance

Confucius

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COMMON ABBREVIATIONS

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AC	Adenylyl cyclase
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ARBD	Alcohol-related birth defects
ARND	Alcohol-related neurological disorders
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2 protein
Bcl-xL	B-cell lymphoma-extra-large protein
Aβ	β-amyloid peptide
BAC	Blood alcohol concentration
BDNF	Brain-derived neurotrophic factor
BrdU	Bromodeoxyuridine
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CNS	Central nervous system
COX-2	Cyclooxygenase-2
P450 2E1	Cytochrome P450 2E1
CREB	Responsive element binding protein
cAMP	adenosine 3',5'-cyclic monophosphate
Cdk5	Cyclin-dependent kinase 5
CA1	Cornu ammonis 1
DG	Dentate gyrus
DNA	deoxyribonucleic acid
DCX	Doublecortin
DYRK1A	Dual specificity tyrosine phosphorylation regulated kinase 1A
EC	Epicatechin
ECG	Epicatechin-3-gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin-3-gallate or epigallocatechin gallate
FAS	Fetal alcohol syndrome
pFAS	Partial fetal alcohol syndrome
FASD	Fetal alcohol spectrum disorders
FGR	Fetal growth restriction
GABA	Gamma-aminobutyric acid
GDs	Gestational days
GDNF	Glial cell-derived neurotrophic factor
GFRα1	GDNF family receptor-α 1
GFAP	Glial fibrillary acidic protein
GSH	Antioxidant molecule glutathione
GSK-3	Glycogen synthase kinase 3
GSK-3β	Glycogen synthase kinase-3β
GAP-43	Growth associated protein 43
HMGB1	High mobility group box 1 protein
HNK-1	Human natural killer-1
H_2O_2	Hydrogen peroxide

OH-	Hydroxyl radicals
iNOS	inducible nitric oxide synthase
IGF	Insulin-like growth factor
IQ	Intelligence quotient
L1	Neural cell adhesion molecule-L1CAM
MDA	Methylenedioxyamphetamine
MRI	Magnetic resonance imaging
MMP-9	Matrix metalloproteinase-9
MAP1B	Microtubule Associated Protein 1B
MAP 2	Microtubule-associated protein 2
MeCP2	Methyl-CpG binding protein 2
MAPK	Mitogen-Activated Protein Kinases
NGF	Nerve growth factor
NCAM	Neural cell adhesion molecule
NeuN	Neuronal nuclei
NT	Neurotrophin
NMDA	N-methyl-D-aspartate
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
Nrf2	Nuclear erythroid 2-related factor 2
NFκB	Nuclear Factor κB
PLGF	Placental growth factor
PNs	Postnatal days
PAE	Prenatal alcohol exposure
ROS	Reactive oxygen species
SD	Standard deviation
SOD	Superoxide dismutase
3D MicroCT	3D Micro-computed tomography
Tlr4	Toll-like receptor 4
Trk	Tropomyosin-related kinase
TrkB	Tyrosine kinase B
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor A
VEGF-R	Vascular endothelial growth factor receptor

ARTICLES THAT MAKE UP THE THESIS

The thesis is structured following the guidelines provided by the University of Barcelona for the PhD degree Doctor in Medicine and Translational Research. It consists of three publications focused on five objectives in a single line of research, two original research papers and a review published in international peer-reviewed journals.

1. In the first article, we assessed the prenatal use of epigallocatechin gallate for the treatment of fetal alcohol spectrum disorders in a mouse model and the effects produced by the two different human drinking patterns on fetuses. The results presented in this publication correspond to objectives 3, 4, and 5 of the thesis.
   Almeida-Toledano L, Andreu-Fernández V, Aras-López R, García-Algar O, Martínez L, Gómez-Roig MD. Epigallocatechin Gallate Ameliorates the Effects of Prenatal Alcohol Exposure in a Fetal Alcohol Spectrum Disorder-Like Mouse Model. *Int J Mol Sci.* 2021;22(2):715.
   Impact factor: 4.556. Quartile: 1st (knowledge area: Biochemistry and molecular biology).

2. In the second article, we evaluated human EGCG bioavailability under different nutritional conditions, covering objectives 1 and 2 of the thesis.
   Andreu-Fernández V, **Almeida Toledano L**, Pizarro N, Navaro-Tapia E, Gómez-Roig MD, de la Torre R, García-Algar O. Bioavailability of Epigallocatechin Gallate Administered with Different Nutritional Strategies in Healthy Volunteers. *Antioxidants (Basel)*. 2020;9(5):E440.
   Impact factor: 5.014. Quartile: 1st (knowledge area: Food science and technology).

3. Finally, a copy of a topical review on murine models in the FASD study is included in the annexes:
   Almeida L, Andreu-Fernández V, Navarro-Tapia E, Aras-López R, Serra-Delgado M, Martínez L, García-Algar O, Gómez-Roig MD. Murine Models for the Study of Fetal Alcohol Spectrum Disorders: An Overview. *Front Pediatr.* 2020;8:359.
   Impact factor; 2.634. Quartile: 1st (knowledge area: Pediatrics).

RESUMEN DE LA TESIS

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Introducción

Según la Organización Mundial de la Salud, la exposición prenatal a alcohol es la principal causa evitable de discapacidad intelectual en el mundo occidental, asociándose a diferentes alteraciones físicas y desórdenes neurológicos que conllevan alteraciones cognitivas y conductuales. El conjunto de estas alteraciones se denominan trastornos del espectro alcohólico fetal (TEAF). Los desórdenes relacionados con esta exposición a alcohol se asocian a importantes problemas en la esfera social y conlleva una serie de costes para el sistema de salud pública. Aunque el consumo del alcohol de forma aguda y compulsiva es el patrón de consumo más habitual, otros tipos de consumo, como el Mediterráneo (bajas dosis de forma continuada), son habituales en determinadas zonas geográficas. Sin embargo, se desconocen actualmente los mecanismos responsables de la toxicidad del alcohol.

Los roedores como la cepa de ratón C57BL/6 permiten evaluar los efectos teratogénicos del alcohol según la dosis y el momento del desarrollo en que se administra, analizando comportamientos complejos similares a los humanos y terapias experimentales prometedoras. Las similitudes en las etapas del desarrollo neurológico en los ratones y humanos permiten llevar a cabo estos estudios, pero hay que tener en cuenta que en el ratón el equivalente al tercer trimestre de gestación humana ocurre en la etapa postnatal. La dismorfología facial debida a la exposición prenatal a alcohol aparece durante el equivalente al primer trimestre de gestación humana, pero el desarrollo neurológico ocurre continuamente durante toda la gestación, por lo que el consumo en cualquier momento del embarazo puede conducir a alteraciones en procesos claves como la proliferación, la migración, la diferenciación, la sinaptogénesis, la gliogénesis, la mielinización y la apoptosis en los linajes neuronales, que conllevan alteraciones neurológicas y del comportamiento.

Por otro lado, el consumo prenatal de alcohol produce deficiencias nutricionales maternas y alteraciones en la angiogénesis e histología placentaria que conllevan a una restricción del crecimiento fetal. Actualmente no disponemos de ningún tratamiento específico para el TEAF, a parte del diagnóstico precoz y el tratamiento de los síntomas. La epigalocatequina galato (EGCG) es un flavonoide con poder antioxidante que se ha evaluado en el tratamiento de diversas patologías humanas. Dado que el alcohol produce un aumento de estrés oxidativo, la EGCG se plantea como una opción terapéutica para el TEAF. Algunos estudios han demostrado que la EGCG se puede distribuir en el cerebro embrionario y fetal mediante su administración a la gestante, pero es necesario conocer mejor su perfil farmacocinético. Actualmente no existe consenso

sobre la concentración más adecuada de EGCG para obtener unos efectos terapéuticos óptimos. Conocer bien el perfil farmacocinético de la EGCG puede ser útil con el fin de utilizarlo en futuros ensayos clínicos.

Hipótesis

La exposición prenatal a alcohol produce diferente grado de restricción de crecimiento fetal, trastornos placentarios y desórdenes neurológicos según el patrón de consumo humano (agudo o *binge versus* moderado crónico o Mediterráneo). La terapia con EGCG administrada en las condiciones farmacocinéticas óptimas puede atenuar estas anomalías.

Objetivos

Los objetivos principales de esta tesis doctoral son evaluar el efecto protector de la terapia con EGCG en un modelo de TEAF en ratón de acuerdo con los dos patrones de consumo humano (agudo o *binge versus moderado crónico o* Mediterráneo), y analizar el perfil de biodisponibilidad específico de la EGCG en humanos bajo diferentes condiciones nutricionales.

Métodos

Para llevar a cabo los objetivos de esta tesis se realizó un estudio preclínico en modelo de ratón y un estudio prospectivo cruzado en humanos.

En el estudio preclínico se utilizó la cepa de ratón C57BL/6. Se realizaron los apareamientos y el diagnóstico de gestación se llevó a cabo mediante la visualización de tapón espermático. Las ratonas gestantes fueron asignados aleatoriamente a 6 grupos experimentales: (1) control Mediterráneo (1,38 mg/kg maltodextrina); (2) etanol Mediterráneo (0,75 mg/kg etanol); (3) etanol Mediterráneo + antioxidante (0,75 mg/kg etanol + 30 mg/kg EGCG); (4) control binge (5,52 mg/kg maltodextrina); (4) etanol binge (3 mg/kg etanol); (6) etanol binge + EGCG (0,75 mg/kg etanol + 30 mg/kg EGCG). Las gestaciones se finalizaron mediante cesáreas el día 19 y se realizaron necropsias con obtención de placenta y tejido cerebral. Se analizó el crecimiento fetal, se realizó estudio de los fenómenos de angiogénesis en placenta (VEGF-A, PLGF y VEGF-R), del estrés oxidativo (Nrf2), y los procesos del desarrollo neurológico que incluyen la maduración (NeuN, DCX), diferenciación (GFAP) y plasticidad neuronal (BDNF) mediante técnicas de Western blot e inmunohistoquímica. Por último, se realizaron los análisis estadísticos correspondientes.

En el estudio cruzado, se reclutaron prospectivamente diez voluntarios sanos (cinco mujeres y cinco hombres). Se realizaron tres series de experimentos clínicos con un período de descanso de siete días entre cada uno: (1) Teavigo® (extracto de EGCG) en ayunas; (2) Teavigo® con un desayuno estándar; y (3) FontUp® (Teavigo® en un

preparado comercial con suplementos nutricionales). Se realizaron extracciones de sangre en los minutos 0, 30, 60, 90, 120, 180, 240 y 360 después de la administración de EGCG. La determinación de EGCG en plasma se realizó mediante el método analítico de cromatografía y espectrometría de masas UPLC-ESI-MS/MS. Se analizaron estadísticamente las siguientes variables farmacocinéticas: AUC_{0-360}, C_{max}, C_{av}, C_{min}, $T_{1/2}$ y T_{max}.

Resultados principales

Respecto al estudio en el modelo animal objetivamos que cualquier patrón de consumo de alcohol (Mediterráneo o *binge*) produce efectos no deseados en la descendencia. Con la administración de dosis elevadas de alcohol encontramos una restricción de crecimiento fetal importante, sin embargo, las dosis más moderadas no fueron suficientes para sobrepasar el umbral requerido para que se produjera un déficit de crecimiento. Cualquier patrón de consumo produjo desequilibrios en la expresión de los factores angiogénicos en placenta (VEGF-A, VEGF-R) y desórdenes en los procesos del desarrollo neuronal, incluyendo la pérdida de neuronas maduras (NeuN), retraso en la maduración neuronal (DCX) y desórdenes en la diferenciación astrocitaria (GFAP). La exposición a alcohol continuada durante el desarrollo fetal puede conducir a una habituación en determinados procesos como la plasticidad neuronal (BDNF). El tratamiento con EGCG fue capaz de revertir las alteraciones del crecimiento fetal y desarrollo placentario, el estrés oxidativo, y los desórdenes en los procesos del desarrollo neurológico fetal.

En el estudio cruzado para el análisis de la biodisponibilidad de la EGCG, con la administración de Teavigo® en ayunas se obtuvieron concentraciones más elevadas en plasma (evaluadas mediante AUC_{0-360}, C_{max} y C_{av}), tanto en hombres como en mujeres. Sin embargo, la variabilidad interindividual fue menor ($T_{1/2}$ más elevada) con la administración de Teavigo® con un desayuno estándar y principalmente con el suplemento alimenticio FontUp®.

Conclusiones

Los resultados presentados en esta tesis apoyan el uso de la EGCG como un potencial tratamiento prenatal para las alteraciones en los procesos neuronales fetales, el desarrollo placentario y el crecimiento fetal producidas por la exposición prenatal a alcohol. Estos hallazgos suponen el punto de partida para plantear nuevas investigaciones sobre el efecto de la EGCG sobre los trastornos del comportamiento relacionados con el TEAF en el adulto. El conocimiento sobre las características farmacocinéticas de la EGCG permitirá su uso en la población gestante humana como

una alternativa segura para el tratamiento de los problemas de salud derivados del consumo prenatal de alcohol.

INTRODUCTION

1. INTRODUCTION

1.1. Alcohol and pregnancy

Alcohol is the most known teratogen. Its frequent use during pregnancy affects normal development of human fetuses promoting severe developmental alterations causing a wide range of physical, behavioral, cognitive, and neurological impairments. In 1968, Lemoine *et al.* established an association between prenatal alcohol exposure (PAE) with certain neurodevelopmental disabilities[1]. However, it was not until 1973 when Jones and Smith provided a first characterization of fetal alcohol syndrome (FAS)[2], defining it as growth restriction, facial dysmorphologies (wide-spaced eyes, mid-facial hypoplasia, and a smooth philtrum), and central nervous system (CNS) disorders, resulting in motor, cognitive and behavioral disorders[3]. Subsequent observational studies identified and characterized the umbrella term fetal alcohol spectrum disorder (FASD)[4] that includes: FAS (the most deleterious manifestation of FASD), partial FAS (pFAS) (an intermediate phenotype defined by the absence of some FAS characteristics), alcohol-related birth defects (ARBD) (certain physical impairments are exhibited), and alcohol-related neurological disorders (ARND) (behavioral and learning neuropsychological alterations, usually without facial dysmorphology[5]) (see Table 1)[5].

Table 1. Updated criteria for the diagnosis of Fetal Alcohol Spectrum Disorders[5]

DIAGNOSTIC CATEGORIES	
FAS	With or without documented prenatal alcohol exposure A diagnosis of FAS requires all features, A–D: A. A characteristic pattern of minor facial anomalies, including ≥ 2 of the following: 1. Short palpebral fissures (≤ 10th centile) 2. A thin upper lip vermilion border (rank 4 or 5 on a racially normed lip/philtrum guide, if available) 3. Smooth philtrum (rank 4 or 5 on a racially normed lip/philtrum guide, if available) B. Prenatal and/or postnatal growth deficiency 1. Height and/or weight ≤ 10th centile (plotted on a racially or ethnically appropriate growth curve, if available) C. Deficient brain growth, abnormal morphogenesis, or abnormal neurophysiology, including ≥ 1 of the following: 1. Head circumference ≤ 10th percentile 2. Structural brain anomalies 3. Recurrent non-febrile seizures (other causes of seizures having been ruled out) D. Neurobehavioral impairment* 1. For children ≥ 3 years of age (a or b): a. With cognitive impairment: – Evidence of global impairment (general conceptual ability ≥ 1.5 SD below the mean, or performance IQ or verbal IQ or spatial IQ ≥ 1.5 SD below the mean) OR – Cognitive deficit in at least one neurobehavioral domain ≥ 1.5 SD below the mean (executive functioning, specific learning impairment, memory impairment or visual-spatial impairment) b. With behavioral impairment without cognitive impairment: – Evidence of behavioral deficit in at least one domain ≥ 1.5 SD below the mean in self-regulation impairments (mood or behavioral regulation impairment, attention deficit, or impulse control)

	2. For children < 3 years of age: – Evidence of developmental delay ≥ 1.5 SD below the mean
pFAS	For children with documented prenatal alcohol exposure, a diagnosis of PFAS requires features A and B: A. A characteristic pattern of minor facial anomalies, including ≥ 2 of the following: 1. Short palpebral fissures (≤ 10th centile) 2. Thin upper lip vermilion border (rank 4 or 5 on a racially normed lip/philtrum guide, if available) 3. Smooth philtrum (rank 4 or 5 on a racially normed lip/philtrum guide, if available) B. Neurobehavioral impairment* 1. For children ≥ 3 years of age (a or b): a. With cognitive impairment: – Evidence of global impairment (general conceptual ability ≥ 1.5 SD below the mean, or performance IQ or verbal IQ or spatial IQ ≥ 1.5 SD below the mean) OR – Cognitive deficit in at least one neurobehavioral domain ≥ 1.5 SD below the mean (executive functioning, specific learning impairment, memory impairment or visual-spatial impairment) b. With behavioral impairment without cognitive impairment: – Evidence of behavioral deficit in at least one domain ≥ 1.5 SD below the mean in self-regulation impairments (mood or behavioral regulation impairment, attention deficit, or impulse control) 2. For children < 3 years of age: – Evidence of developmental delay ≥ 1.5 SD below the mean For children without documented prenatal alcohol exposure, a diagnosis of PFAS requires all features, A–C: A. A characteristic pattern of minor facial anomalies, including ≥ 2 of the following: 1. Short palpebral fissures (≤ 10th centile) 2. Thin upper lip vermilion border (rank 4 or 5 on a racially normed lip/philtrum guide, if available) 3. Smooth philtrum (rank 4 or 5 on a racially normed lip/philtrum guide, if available) B. Growth deficiency or deficient brain growth, abnormal morphogenesis, or abnormal neurophysiology 1. Height and/or weight ≤ 10th centile (plotted on a racially or ethnically appropriate growth curve, if available) OR 2. Deficient brain growth, abnormal morphogenesis or neurophysiology, including ≥1 of the following: a. Head circumference ≤ 10th percentile b. Structural brain anomalies c. Recurrent non-febrile seizures (other causes of seizures having been ruled out) C. Neurobehavioral impairment* 1. For children ≥ 3 years of age (a or b): a. With cognitive impairment: – Evidence of global impairment (general conceptual ability ≥ 1.5 SD below the mean, or performance IQ or verbal IQ or spatial IQ ≥ 1.5 SD below the mean) OR – Cognitive deficit in at least one neurobehavioral domain ≥ 1.5 SD below the mean (executive functioning, specific learning impairment, memory impairment or visual-spatial impairment) b. With behavioral impairment without cognitive impairment: – Evidence of behavioral deficit in at least 1 domain ≥ 1.5 SD below the mean in self-regulation impairments (mood or behavioral regulation impairment, attention deficit, or impulse control) 2. For children < 3 years of age: Evidence of developmental delay ≥ 1.5 SD below the mean
ARND	Requires features A and B (definitive diagnosis cannot be made in children < 3 years of age): A. Documented prenatal alcohol exposure B. Neurobehavioral impairment* For children ≥ 3 years of age (a or b): a. With cognitive impairment:

	– Evidence of global impairment (general conceptual ability ≥ 1.5 SD below the mean, or performance IQ or verbal IQ or spatial IQ ≥1.5 SD) OR – Cognitive deficit in at least two neurobehavioral domains ≥ 1.5 SD below the mean (executive functioning, specific learning impairment, memory impairment or visual-spatial impairment) b. With behavioral impairment without cognitive impairment: – Evidence of behavioral deficit in at least two domains ≥ 1.5 SD below the mean in self-regulation impairments (mood or behavioral regulation impairment, attention deficit, or impulse control)
ARBD	Requires features A and B: A. Documented prenatal alcohol exposure B. One or more specific major malformations demonstrated in animal models and human studies to be the result of prenatal alcohol exposure: cardiac: atrial septal defects, aberrant great vessels, ventricular septal defects, conotruncal heart defects; skeletal: radioulnar synostosis, vertebral segmentation defects, large joint contractures, scoliosis; renal: aplastic/hypoplastic/dysplastic kidneys, "horseshoe" kidneys/ureteral duplications; eyes: strabismus, ptosis, retinal vascular anomalies, optic nerve hypoplasia; ears: conductive hearing loss, neurosensory hearing loss

*Adaptive skills should be assessed, but such deficits cannot stand alone for diagnosis.

Thus, there is a relationship between behavioral deficits in FASD subjects with structural changes in brain organogenesis: the *corpus callosum* may lose its structure (agenesis) and generate cognitive deficits linked to attention, executive and psychosocial functions, language and reading comprehension[6]; the cerebellum and anterior part of the vermis may suffer hypoplasia and affect motor skills and learning capacity[7]. Moreover, asymmetry of the hippocampus in FAS children may also affect their memory[8]. The degree of structural abnormalities in the brain correlates with the severity of FAS-like facial features, and in turn, with more serious behavioral problems[9]. Human socialization begins at an early age and continues throughout life. Social behavior in adults is the result of a complex combination of genetics, brain development, childhood experiences, and learning. Changes in social behavior in adults with FAS may be triggered by an interaction of their genetic background, alcohol-induced alterations in specific brain structures, abnormal early socialization processes, and disabilities in social learning processes throughout their life[10].

According to the World Health Organization, PAE is the main preventable cause of intellectual disability in the western world[11–13], with FAS being the most common non-hereditary cause of intellectual disability[14]. A recent meta-analysis estimated global prevalence of alcohol use during pregnancy to be 9.8%[15]. Furthermore, a study carried out in 2018 on Spanish population showed a prevalence of 64.7% in pregnant women for any intake of alcohol in any trimester of pregnancy, although prevalence of FASD in this population was unknown[16]. Alterations grouped within FASD affect 1% of the world's pediatric population and the estimated prevalence of FAS in the general population is 14.6 per 10,000 people[15]. In the USA, the prevalence of FAS is 1.3 to 4.6 per 1,000 births, increasing to 9.1 per 1,000 births for FASD[14]. Based on reports from studies using physical examinations, experts estimate that the full range of FASDs in the USA and

some Western European countries reaches 2 to 5% among school children (or 2 to 7 per 1,000 in the general population)[17,18]. The highest rates of FAS and FASD are found in South Africa, a heterogeneous ethnic population with high prevalence of alcohol abuse, where rates between 68 to 89 per 1,000 births have been described[9,19]. Moreover, increased incidence of PAE and risk of FASD has been reported in children adopted from Eastern European Countries (EEC)[20], FASD reaching a prevalence of 52% in Swedish children adopted from EEC[21]. Although acute and binge alcohol intake is the most common pattern of consumption, other types such as the Mediterranean (continuous low doses), are common in certain geographical areas.

Therefore, PAE-related disorders may lead to major problems within social environments, as well as economic setbacks for the public health system. Many of these costs could be reduced with effective social policies and intervention programs aimed at improving the lives of people with FASD and their families, and the prevention of further alcohol-exposed pregnancies.

1.2. Fetal alcohol spectrum disorders-like animal models

FASD studies in humans have common limitations due to the complexity in correctly measuring certain variables such as maternal diet or health, or the volume and timing of ethanol exposure during pregnancy. It is possible to overcome these difficulties by using animal models, simple, effective and reliable tools for alcohol research. These models are useful for understanding the molecular mechanisms underlying alcohol teratogenicity and monitoring cognitive and behavioral changes. Moreover, animal models allow assessing different therapeutic approaches in preclinical studies, initial screening of compounds, and as a previous phase for future human clinical studies.

The invertebrate *Caenorhabditis elegans* is a simple animal model to study developmental processes and is commonly chosen to study the effects of ethanol on molecular pathways. However, the embryos develop outside the body and precise ethanol concentrations administered cannot be finely controlled[22]. On the other hand, alcohol metabolism differs substantially from that in humans[22]. The zebrafish (*Danio rerio*) has several physiological and genetic similarities with humans[23], which makes it a suitable vertebrate alternative model. There are further advantages regarding the effects of ethanol: extensive knowledge of all developmental stages, short development time, and large number of offspring[24]. Zebrafish eggs and embryos are transparent (just like nematodes), making embryonic development easy to follow, facilitating exposure to alcohol of the embryos during different and precise developmental stages, and easy determination of physical malformations and simple behavioral changes[23,25]. By contrast,

the chorion of the egg acts as a barrier and large volumes of ethanol are necessary to ensure its penetration[24].

Mammals offer significant advantages in the study of brain structures or complex behaviors[26]. Although primates could be the *gold standard*, there are some disadvantages, mainly the long duration of the studies and ethical limitations[26]. Rodents are frequently used in FASD research because they are easy to handle, have a short gestational period, and produce large numbers of offspring. Rats offer the advantages of being larger and show a more sophisticated behavior in comparison to mice. Regardless, mice (particularly the C57BL/6 strain) are the most commonly used mammals due to their ease of care, availability of transgenic and disease models, short lifespan, basic physiology, and genetics similar to that of humans. Teratogenic effects of alcohol exposure in mice have been reported, including craniofacial malformations, altered neurogenesis processes, fetal growth restriction and soft-tissue and skeletal abnormalities[27,28]. The main disadvantage in using rodents for FASD research is that the third trimester equivalent to human development in rodents occurs after birth. Thus, there are differences in the processes of absorption, distribution, metabolism and elimination in rodents in comparison to the human utero, with no influence of the placental barrier. Interestingly, C57BL/6J is the strain with the highest preference for alcohol[29].
In following sections, we discuss details that need to be considered when a murine model is chosen for a FASD study.

1.2.1. Alcohol exposure patterns. Blood alcohol concentrations

Drinking patterns are characterized by the amount and frequency of ethanol taken. This is measured by blood alcohol concentration (BAC) and expressed as weight of alcohol per unit of volume of blood. BAC depends on factors such as dosage, pattern of exposure, metabolic rate, food consumption, tolerance, and genetics[30,31].

In animal models, BAC is defined as the amount of ethanol per unit of blood (usually mg/dL), measured when ethanol reaches the highest concentration in peripheral circulation[32]. In rodents, peak concentration is detected between 30-150 minutes (50-100 minutes in mice and 50-150 minutes in rats) following administration. The timeline of BAC depends on the administration route, dosage, and species (rate of ethanol metabolism is 550 mg/kg/h and 300 mg/kg/h in mice and rats, respectively)[33]. Severe neurotoxicity is typically linked to binge-like episodes causing higher BACs (e.g., BAC > 300 mg/dl in rats).

Kelly et al. showed that binge-like alcohol exposure is more harmful than non-binge exposure in rat brain development with the same dose of ethanol. The authors administered 6.6 g/kg/day of ethanol to neonatal rats using artificial rearing, following one of two possible patterns: a continuous pattern (24 hours per day) for several days,

which resulted in an average BAC peak of 79-97 mg/dL, or an acute exposure pattern (eight hours per day) for the same length of time, resulting in an average BAC peak of 56-415 mg/dL. Lower brain growth was observed in the acute exposure group in comparison to the continuous pattern[34]. Other findings support the hypothesis that lower daily doses of ethanol following a binge-like pattern leads to lower brain weight and cell loss in different brain areas than higher non-binge doses. Three groups of ethanol-exposed rat pups were compared. One group was exposed to 4.5 g/kg/day in a condensed pattern (four hours per day), the second group was exposed to the same dose although administered in a less condensed pattern (eight hours per day), and the third group was exposed to a higher dose of alcohol (6.6 g/kg/day) administered in a continuous pattern (24 hours per day). The resulting average BAC peaks were 361 mg/dL, 190 mg/dL, and 39 mg/dL, respectively. The authors found that pups exposed to 4.5 g/kg/day over four hours had the lowest brain weight, followed by the second group. The animals that ingested highest doses of ethanol throughout 24 hours had the highest brain weights[35]. These findings demonstrate that binge-like pattern ethanol intake is more harmful than higher doses taken for longer periods due to higher BAC peaks for shorter periods. It is also important to mention that continuous alcohol exposure, reaching lower BAC levels (i.e., BAC < 40 mg/dl in rats) despite higher doses, induces more subtle brain injuries[34,35].

1.2.2. Control group

Several studies have assessed the influence of nutritional intake on the teratogenic effects of alcohol[36,37]. Alcohol can replace other nutrients because of its caloric content and may interfere with the absorption of other nutrients due to its inflammatory effects on the stomach[38].

Pair-fed control has been used in some FASD-like animal model experiments, since it acts as a calorie-matched control group. A carbohydrate substance (e.g., maltose dextrin or sucrose) is usually employed to replace ethanol-derived calories in the diet[39]. A pair-fed group may also allow monitoring stress conditions. On the other hand, the pair-fed group is considered an imperfect control group, since the pattern of food consumption in this group is different from a physiological intake. Individuals in pair-fed controls consume the assigned food as soon as it is available, creating additional stress associated to food restriction. In addition, in the pair-fed group it is not possible to match the effect of alcohol on the absorption of other nutrients because of its inflammatory effects. Thus, some researchers have suggested the use of a control group (baseline) known as non-handled, *ad libitum*, or *sham*, in which the intake of nutrients resembles the physiological one. This is useful to avoid biases caused by ethanol interference in nutrient absorption[40]. Consequently, the use of a pair-fed group and an *ad libitum* control

group should be considered as an alternative when designing a FASD murine model study.

1.2.3. Routes of administration and dosage forms

Several modes of ethanol administration methods have been described, particularly in rodent gestation. Ethanol delivery methods directly affect variables such as the alcohol exposure pattern, exact amount of alcohol taken, and generated stress. These variables must be taken into account during the experimental design. Voluntary ethanol feeding and intragastric gavage are the most physiological administration methods. Voluntary ethanol feeding[41,42] is a safe technique when low stable BAC levels want to be reached. Conversely, intragastric gavage[40] offers a more accurate control of doses and timing, and higher BACs. Inhalation[43] or injection[44] offer some advantages compared to voluntary ethanol drinking and intragastric gavage due to their time efficiency. Artificial rearing is a useful method when the aim of the study is alcohol administration in a third trimester equivalent model[40,45,46]. Briefly, the choice of method must consider the purpose of the experiment and the researcher´s experience. Table 2 (based on a previous review[47]) summarizes the characteristics of the main routes of alcohol administration in rodents and dosage forms, focusing mainly on mice.

Table 2. Characteristics of the different routes of ethanol administration in mice. Own authorship.

ADMINISTRATION ROUTE	CHARACTERISTICS	REACHED BAC	ADVANTAGES	DISADVANTAGES
VOLUNTARY ETHANOL FEEDING	Oral, self-administration. Pre-gestational alcohol consumption is usually introduced[48]. Sometimes, ethanol is added to flavored liquid nutritional formulas (Liquid-diet or Sustacal) to allow easy self-administration[49,50]. 10-20% (vol/vol) ethanol solution[48]. Possibility of isovolumetric and isocaloric pair-fed diet (e.g., maltose-dextrin) in controls[39]. Drinking in the dark (DID) mimics a binge-like pattern[51]	50-100 mg/dL when ethanol intake is 1-2 g/kg [10% (vol/vol) ethanol solution]	Prevents the stress caused by other invasive methods. Safe technique. Easy to carry out. Gradual BAC increase. Low, stable BAC levels. Used prenatally	Lower ethanol BAC are achieved in comparison to other administration routes. Not useful for binge drinking pattern. Difficult control of dose and timing. No proper control of dose in breastfeeding pups. Not recommended postnatally. Lower BAC achieved if saccharin or a sucrose-sweetened solution is added to the alcohol
INTRAGASTRIC GAVAGE	Administration of ethanol into the stomach using a gavage needle. Administered volumes < 2 mL/100 kg body weight[52]. Allowed alcohol concentration < 31.5% (vol/vol)[52]. Ethanol dose 2-6 g/kg/day[39].	250-300 mg/dL (60 minutes) for administration doses of 3.8 g/kg [21% (wt/vol) ethanol solution]	Useful for binge drinking pattern. Accurate control of dose and timing. Reliable high BAC. Useful for pre- and postnatal administration	Inhibition of suckling behavior in neonates. Stressful procedure for animals. Invasive procedure

	Ethanol vehicle (water, saline solution or nutritional formula)[39]			
INHALATION	Inhalation chamber filled with ethanol vapor[53]. Sometimes, administration of pyrazole to obtain stable BACs[43,54]	150-250 mg/dl when volatized ethanol (ethanol 95%) is delivered into the chamber at a rate of 10 liters/min	Reliable high BACs. Not a stressful technique for animals. Time and labor efficient. Useful for pre- and postnatal administration. Higher BACs in neonates than in mothers	Does not mimic human intake routes. Special equipment required. Interindividual variations
INTRAPERITONEAL INJECTION	Ethanol solution injection into the intraperitoneal space[55]. Single or multiple doses for several days during pregnancy	350-400 mg/dL (60 minutes) for administration doses of 3.8 g/kg [21% (wt/vol) ethanol solution]	Rapid increase in BACs. Time efficient. Useful for pre- and postnatal administration. Useful for binge drinking pattern	Handling-induced stress. Different to human intake routes.. This administration route produces higher BACs in fetuses than other routes using the same PAE. Higher incidence of malformations when used during first trimester equivalent.
ARTIFICIAL REARING	Intragastric gavage ethanol discharge in pups while being kept in a special setting to mimic maternal environment[40,45,56]. Placement of gastrostomy catheters.	150 mg/kg when ethanol solution of 2,5 g/kg is administered or 420 mg/kg when ethanol solution of 7,5 g/kg is administered*	Accurate control of dose and timing. Useful for postnatal administration. Mimics human third trimester	Invasive and expensive technique. Social factors removed due to isolation of pups.

1.3. Effects of alcohol on embryonic mouse brain developmental stages

During the development of the CNS throughout pregnancy, there are vulnerable periods sensitive to environmental insults. PAE affects brain organogenesis differently depending on the dosage, timing, developmental stage (moment), and location of the cell types involved in the biological stages (Figure 1). Key processes such as proliferation[57], migration[58], differentiation[59], synaptogenesis[60,61], gliogenesis, myelination[62], and apoptosis[63,64] are altered by PAE leading to congenital abnormalities and functional deficits in the CNS during fetal development (Figure 2)[65,66].

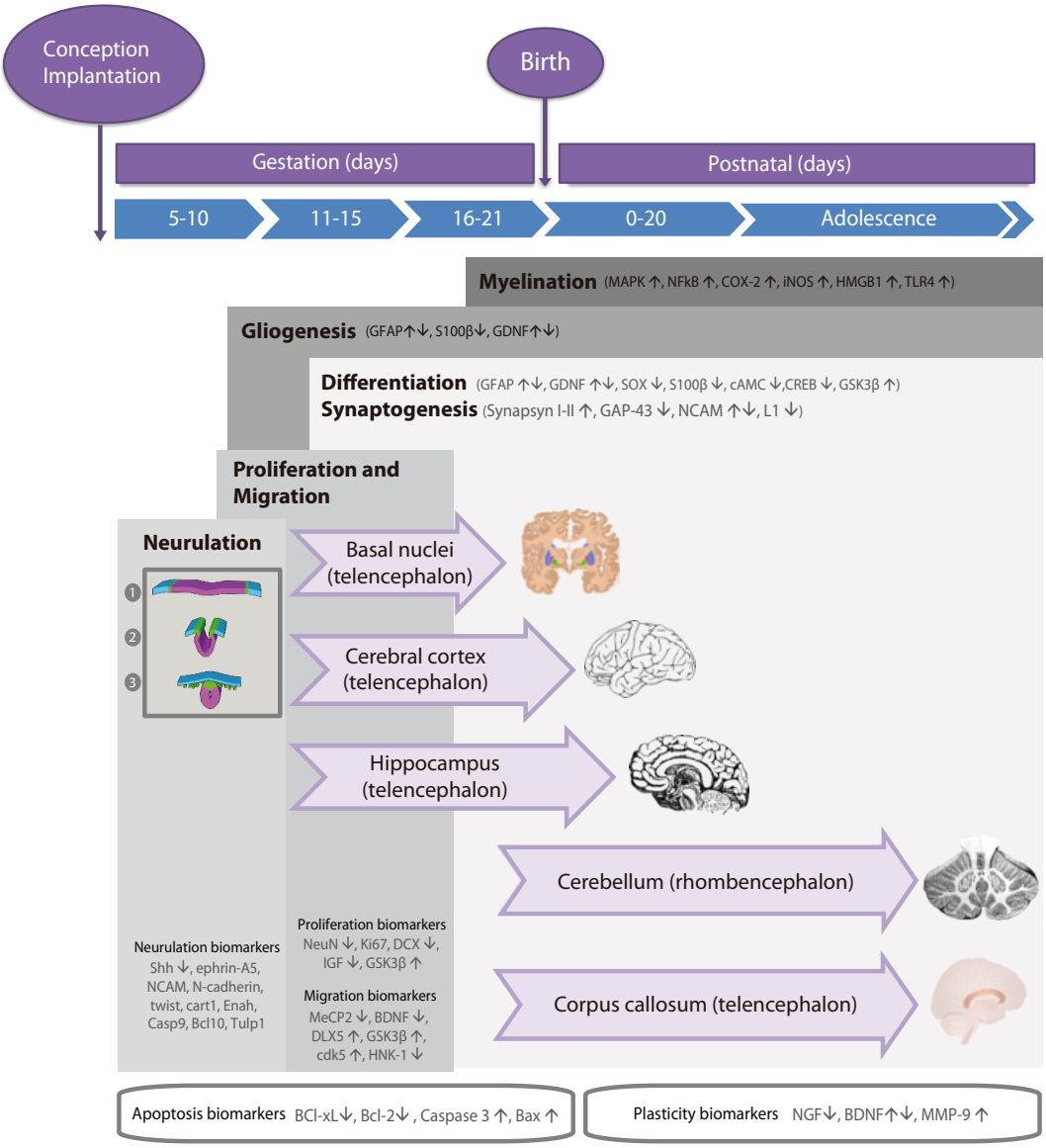

Figure 1. Timeline of neurodevelopmental processes and brain neurogenesis in rodent embryos by areas sensitive to alcohol injury. Proteins in neurulation, proliferation, migration, differentiation, synaptogenesis, gliogenesis, and myelination neurodevelopmental processes. Changes in the levels of these biomarkers (up- or down-regulation) caused by prenatal alcohol exposure are represented by arrows. Stages of neurulation: (1) Neuroectodermal tissues differentiate from the ectoderm and thicken into the neural plate. The neural plate border separates the ectoderm from the neural plate. (2) The neural plate bends dorsally, with the two ends eventually joining at the neural plate borders, forming the neural crest. (3) The closure of the neural tube disconnects the neural crest from the epidermis. Neural crest cells differentiate to form the peripheral nervous system. Own authorship.

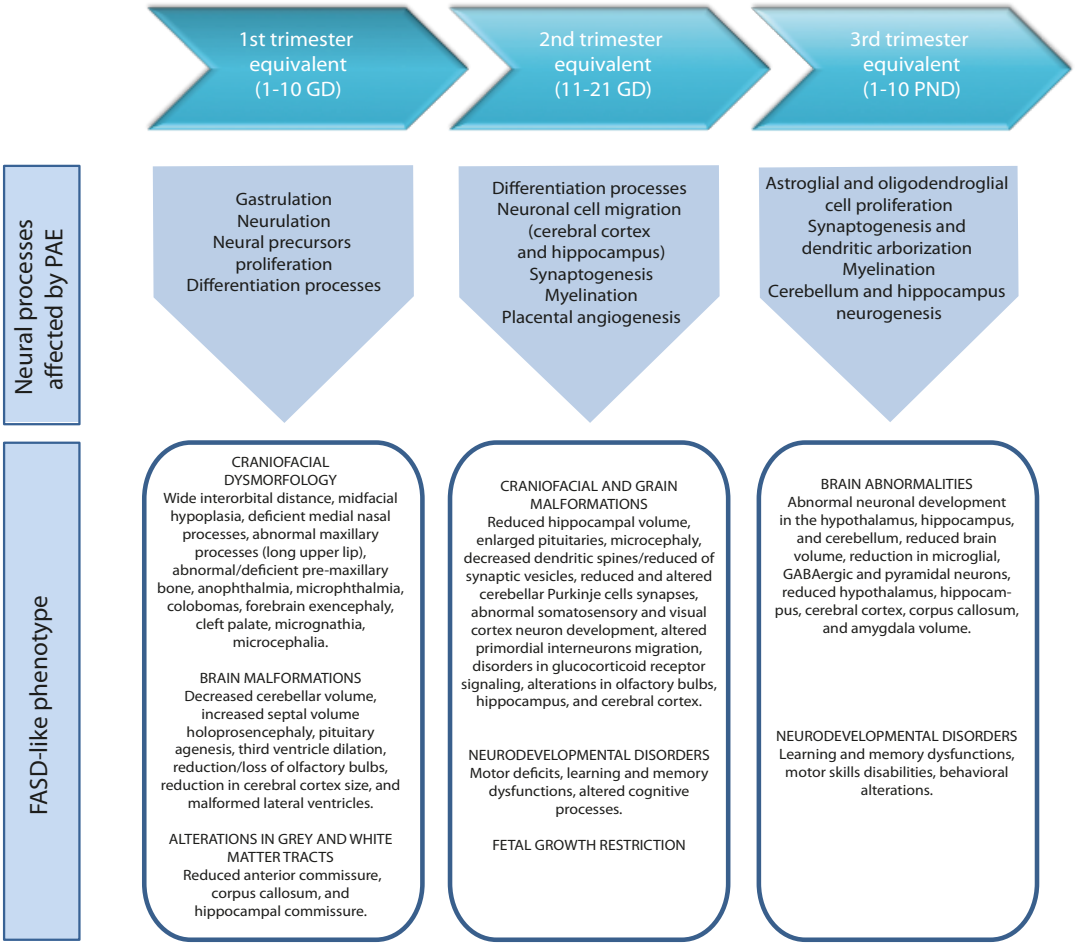

Figure 2. Harmful effects of prenatal alcohol exposure in mice according to human trimesters equivalents[67]. First trimester equivalent: cranial dysmorphologies[68,69], brain malformations[68,70], and altered grey and white matter tracts[71]. Second trimester equivalent: craniofacial and brain malformations[70,72,73], neurodevelopmental disorders[74,75], and fetal growth restriction[76,77]. Third trimester equivalent: brain abnormalities[78–80], neurodevelopmental disorders[78,81]. GD: gestational day. PND: postnatal day. Own authorship.

The anatomies of human and rodent brains show analogous structures and similar stages of development, but exhibit some anatomical and functional differences. Human pregnancy consists of three prenatal trimesters in which the brain rapidly grows between weeks 25 and 38. Several differentiation and proliferations processes occur in the third trimester of gestation, with maximum brain growth rate at birth and gradual decrease in early life[67,82]. Pregnancies in rats and mice are shorter than in humans (rats: 21–23 days; mice: 20–22 days) and newborns undergo substantial brain development following birth[62,63]. The first[67] and second equivalent trimesters in a human pregnancy correspond to gestational days (GDs) 1–10/11 and 11–21/22, respectively, in rats and mice (mice usually give birth on GD 21 and rats on GD 22). The third trimester equivalent in humans correlates to postnatal days (PNDs) 1–10. The ontogeny of specific behaviors

may be used to draw inferences regarding the maturation of specific brain structures or neural circuits in rodents and humans. Despite the similarities between human and rodent brain development it is important to consider that rodents do not exactly mimic the developmental phases of human gestation (Figure 1).

Cellular precursors of the brain and the spinal cord develop through neurulation in early embryogenesis (Figure 1). The cellular fate of neurulation is the formation of the notochord, which defines the primitive axis of the embryo and determines the vertebral system. The neural tube closure starts in the hindbrain area above the origin of the notochord, and continues anteriorly and posteriorly, making a caudal-to-rostral gradient in the developing brain. Neural tube formation finishes at GD 10–11 in rodents[83]. Early in the second week of pregnancy (GD 7 in mouse, GD 9.5 in rats), neurogenesis and subsequent cell migration shape specific areas of the CNS in the forebrain, midbrain, and hindbrain, promoting distinct series of developmental processes[83]. Therefore, the first critical developmental stage for PAE occurs between GD 5 and 11, implying alterations in organogenesis, neural tube formation, and proliferation of neuronal precursors in areas adjacent to the neural tube. High levels of alcohol exposure during this stage not only cause major neural tube defects, but also lead to facial dysmorphologies similar to those observed in children affected with FAS.

The second critical developmental stage occurs between GD 11 and 21. During this period, most CNS areas are involved in distinct differentiation processes and several neuronal cell types emerge and migrate to specific areas of the brain (including the cerebral cortex and the hippocampus) (Figure 1). The developmental phase of the different cell lineages varies according to their spatial location in distinct brain areas. PAE particularly affects the neurulation, proliferation and migration processes of the neocortex, cerebellum, hippocampus, and the basal ganglia. The last decisive developmental period occurs from GD 18 to PND 9 and is characterized by the proliferation of astroglial and oligodendroglial cells, synaptogenesis, and dendritic arborization, which produce an increase in brain weight. At the same time, neurogenesis continues in the cerebellum and the dentate gyrus (DG) of the hippocampus. Alcohol exposure during the third trimester induces severe neuronal loss, reactive gliosis, impaired myelination, as well as damage to the prefrontal cortex, and hippocampal and cerebellar regions[72,84,85].

1.3.1. Proliferation

Neurogenesis is a highly regulated process whose timing and phases depend on the anterior-posterior gradient in the neuronal axis and regions of the brain formed during organogenesis. Most cell proliferation processes take place throughout all

neurodevelopment stages[86], although the most expansive phase occurs in the second half of pregnancy (mice: GD 10-21, rats: GD 11-22). This is a key developmental period due to the vulnerability of neuronal precursors and brain structures to ethanol toxicity[57], which may cause permanent alterations and profound behavioral deficits. For that reason, the effect of PAE on proliferation and differentiation processes are assessed not only during fetal development but also later in life. As shown in Figure 1, several biomarkers help identify and evaluate neurogenesis and proliferation processes during neurodevelopment.

NeuN is expressed in nearly all postmitotic neurons, representing a reliable marker of mature neurons[87]. This protein may also act as a biomarker of neuronal integrity, as it decreases in brain regions, e.g., the hippocampus, following ethanol exposure in rats[87–89]. Ki67 has been thoroughly analyzed as a proliferation biomarker during neurogenesis in PAE studies.

Several groups have studied the effect of PAE on distinct hippocampal regions, with different results. Reduced number of granular cells in the DG and pyramidal cells have been reported in specific regions of the rat hippocampus after GD 1-20 plus PND 4-10 of ethanol exposure[35,90], and on the third trimester equivalent[35,89,90], with no changes in the number of hippocampal neurons with ethanol exposure during GD 1-20[90,91]. Komada *et al*. showed a reduced proliferation rate (measured by Ki67) in mouse telencephalon after PAE on GD 6-18[92]. Conversely, West *et al*. report an increase in the number of granular cells of the DG in rat hippocampus after ethanol exposure during the third trimester equivalent[93]. Thus, early disturbances in proliferation after PAE may differ depending on the developmental period at which ethanol exposure occurs.

Some of the effects of PAE on the hippocampus can be identified from birth, but others are more subtle and difficult to detect in early stages. The consequences of PAE on hippocampal cell proliferation and survival in young adult animals are not always persistent[40,46,94]. Interestingly, no changes in hippocampal cell proliferation (assessed by Ki67 and BrdU), but an increase in immature neurons of adult hippocampus in rats prenatally exposed to alcohol, have been described, probably due to a compensatory mechanism against PAE effects[40]. Other authors have shown alterations in cell proliferation (measured by Ki67[46] and BrdU[46,94]) and increased neuronal maturation in the DG of the hippocampus in young adult rats prenatally exposed to ethanol. More recently, Mohapel-Gil *et al*. described significant decreases in adult hippocampal neurogenesis in aged rats after PAE (during the first and second trimester equivalent), not previously seen in younger animals. These findings suggest a more conserved neurogenesis capacity in early stages of life[95]. Moreover, Delatour *et al*. analyzed Ki67 levels in pyramidal cells in adolescent mice exposed to ethanol on GD 13.5-16.5, and

showed there were no changes when compared to controls[96]. Once again, it seems that alterations in hippocampal neurogenesis vary according to the timing of ethanol exposure.

Coleman *et al*. examined the long-term effects on adult hippocampal neurogenesis after ethanol exposure in PND 7 in male and female mice. Increased Ki67 levels were found in the DG in males but not in females[80]. This reveals gender differences regarding susceptibility to PAE.

PAE also affects the activity of enzymes involved in neurogenesis and proliferation, promoting hippocampal function behavioral disorders. Glycogen synthase kinase-3β (GSK-3β) is highly expressed during brain development (from GD18 to PND10 in rats[97] and GD16 to PD18 in mice[98]), modulating different developmental processes such as neurogenesis, differentiation, and neuronal survival. GSK-3β activation sensitizes neurons to ethanol-induced injury, deregulating cell proliferation mechanisms[99]. Increased levels of GSK-3β post-PAE activates apoptosis in neural progenitor cells, decreasing neurogenesis and differentiation in immature brains. Additionally, ethanol reduces insulin-like growth factor (IGF) receptor signaling, affecting neural proliferation and decreasing the transcription of c-myc, c-fos, and c-jun51 in cell cultures[100].

Current evidence indicates that prenatal and neonatal alcohol exposure reduces the number of mature and immature neurons. Interestingly, this reduction is subtle when ethanol exposure is not continuous. Nonetheless, brain region, developmental stage, and cell type are key factors when analyzing proliferation processes.

1.3.2. Migration

Migration from ventricular and germinal layers occurs radially in the medial/dorsal neocortex and tangentially in other regions of the forebrain[101]. On GD 5, superficial layers are still not clearly defined[102]. On GD 14.5 (mice) or GD17 (rats), first cell lineages reach the area that will form the laminae of the cortical plate. Throughout the rest of the gestation period until adulthood, the cortical plate gets thicker and more cells migrate from the ventricular zone[103]. When proliferation is disrupted, migration is also affected (Figure 1). PAE alters proliferation and migration[58], affecting neural crest migration and causing cytoskeletal rearrangements. These phenomena destabilize the formation of focal adhesions in cell lineages, reducing their capacity for directional migration. Moreover, the activity of glycogen synthase kinase 3 (GSK-3) and cyclin-dependent kinase 5 (cdk5) modulate microtubule-associated protein 1B (MAP1B) phosphorylation, involved in the regulation of microtubules and actin filaments in neurons needed in migration processes[104]. In vitro, ethanol inhibits neurite outgrowth by activating GSK-3β[105]. Conversely, PAE promotes GABAergic interneuron migration by inducing epigenetic alterations in the methylation pattern of the MeCP2-BDNF/DLX5 pathway.

MeCP2 regulates expression of the brain-derived neurotrophic factor (BDNF), a marker of neuronal plasticity and cellular survival known to influence GABAergic interneuron migration[106]. MeCP2 has been shown to regulate DLX5 transcription, a transcription factor involved in the migration and maturation of GABAergic interneurons in mouse models[107]. The human natural killer-1 (HNK-1) carbohydrate is also used as a biomarker in migration processes studies involving cranial neural crest cells[108]. Results indicate reduced levels of HNK-1 in a model of chick embryos exposed to 2% ethanol, which suggests that PAE may disrupt cranial neural crest cell migration.

Long-term effects of PAE on migration have also been evaluated. Miller *et al*. describe the harmful effects of alcohol on proliferation and migration in rats prenatally exposed to alcohol. The authors found a delay in migration of early and late-generated neurons in rats following PAE between GD 6 and GD 21. Ethanol blocks neuronal migration probably by leading to a desynchronization of cortical development that interferes with the establishment of a normal neural network[58]. Skorput *et al*. studied the effects of PAE on GABAergic interneurons in mice. They found an increase in BrdU labelling in the medial ganglionic eminence showing an increase in neurogenesis, as well as an increase in parvalbumin-expressing GABAergic interneurons in the medial prefrontal cortex in adults. These findings support the contribution of GABAergic interneuron migration disorders to persistent alterations in cortical development in adulthood[109].

In summary, migration is a set of complex processes regulated by different molecular pathways that are disrupted in several checkpoints when ethanol exposure occurs.

1.3.3. Differentiation

Processes of neuroblast differentiation initiate after neuronal precursors have completed their last division and are ready to migrate to a specific area[110–112]. Depending on the fate (brain area) of migration, neuronal precursors will differentiate into neurons, astrocytes, or oligodendrocytes[113]. The differentiation of the cerebral cortex implies the formation of laminae in the radial domain from the ventricular zone to the pial surface and the subdivision of functional areas in the tangential domain, in rostrocaudal and mediolateral axes. During this process, the laminar fate is determined by cell-to-cell interactions and cell autonomous restriction[110].

Several proteins, used as biomarkers, are involved in the differentiation processes. Doublecortin (DCX) has been studied in depth as an endogenous marker of immature neurons. The effects of prenatal chronic ethanol consumption on adult neurogenesis (PND 56) has been assessed in C57BL/6J mice, revealing a decrease of DCX in the hippocampus after PAE[88]. Quantification of immature neurons labelled with DCX in

mouse was lower in the group of individuals exposed to alcohol in the prenatal period compared to controls. Moreover, DCX levels were lower in males than in females[114]. Broadwater *et al*. obtained similar results after PAE by oral gavage on PND28-48, with decreased DCX levels in the DG of adolescent mice. Furthermore, after interrupting ethanol exposure, less differentiated neurons in adulthood were found in rats[115]. Elibol-Can *et al*. observed slight changes in the number of granular cells labelled with DCX in hippocampal DG on PND 30. The authors reported a decrease in the volume of the hippocampus in rats after a daily ethanol dose of 6 g/kg during the second trimester equivalent[116]. Likewise, Hamilton *et al*. studied the long term-effects of single or continuous exposure to alcohol during the third trimester equivalent in mice and the effect of voluntary exercise as a therapy. Mice were exposed to ethanol on PND 7 or PND 5, 7, and 9 and DCX measured in adulthood. No differences in DCX levels were found in ethanol-exposed groups. Nevertheless, changes were seen in the group exposed to ethanol during PND 5, 7, and 9 with the Rotarod method and passive avoidance behavioral tests, which measure motor coordination and memory, respectively[117]. Conversely, Coleman *et al*. observed increased levels of DCX after ethanol exposure in the DG on PND 7 in adult male mice, but not in females[80].

Long-term effects of PAE have also been studied using other biomarkers. Choi *et al*. assessed the effects of PAE on BrdU levels in adult mice exposed to ethanol during the two first trimester equivalents. No differences in neuronal proliferation nor differentiation were found after evaluating BrdU levels[42]. Boehme *et al*. studied BDNF levels of rats exposed to ethanol during the three trimester equivalents. They found no changes in BDNF levels of animals exposed to ethanol in the prenatal period. However, increased BDNF levels were observed in groups assigned to voluntary exercise[46]. Gil-Mohapel *et al*. reported increases in NeuroD levels in adult rats exposed to ethanol during the three trimester equivalents. Speeding up of differentiation processes is probably due to the increase in immature neurons showed in prenatally exposed groups[40]. The changes observed in the differentiation processes in adult rodents exposed to ethanol during the prenatal period vary depending on the used biomarker. The increase in neuronal differentiation may occur as a compensation of the cellular loss in fetal life.

The cAMP responsive element binding protein (CREB) is directly correlated to neurogenesis, differentiation, neuronal connectivity and plasticity[118]. Ethanol exposure disrupts the activity of adenylyl cyclase (AC), reducing cAMP/CREB signaling and altering the differentiation processes during neurodevelopment[118]. *In vivo* and in vitro studies have shown that acute alcohol exposure enhances agonist-stimulated AC catalytic activity, while chronic alcohol exposure produces adaptive changes in AC[119–

[121]. Additionally, GSK-3β over-expression in neural cells disrupts CNS maturation and the differentiation processes in mouse at PND 60-120[122].

The glial cell-derived neurotrophic factor (GDNF) is a growth factor necessary for the development, differentiation, proliferation, and function of midbrain dopaminergic neurons. The signaling pathway for GDNF is initiated by the binding of GDNF to its co-receptor, GDNF family receptor-α 1 (GFRα1), which leads to the recruitment of the RET receptor tyrosine kinase. The activation of RET promotes the up-regulation of downstream signaling pathways such as ERK1/2[123] and P13K[124], firing the activity of dopaminergic neurons. Moderate administration of alcohol increases GDNF expression, exerting a protective function against PAE. However, after acute (binge) ethanol exposure in rats, GDNF expression decreases and its protective function is diminished[125]. A recent study performed in adult rats exposed to alcohol showed a decrease in DNA methylation as the leading cause of GDNF epigenetic changes following alcohol exposure[126].

Alcohol has deleterious effects on astrocytes despite them being less susceptible than neurons to moderate alcohol consumption[127]. Glial cell alterations due to PAE lead to changes in neuron-glia interactions, which causes developmental defects of the brain[128]. The glial fibrillary acidic protein (GFAP) is a biomarker of mature astrocytes, commonly evaluated in differentiation processes during development. In vitro studies using primary cultures of astrocytes from 21-day old fetuses show initial increased values of GFAP levels post-ethanol exposure[129], although these GFAP values decrease after three weeks[129]. GFAP levels in rat neonates have been shown to increase following ethanol exposure in different brain areas, e.g., the hippocampus, cerebellum, and cortex, as per different administration routes[130–132]. Results in *in vitro* models suggest different effects of ethanol on astrocytes depending on the neurodevelopmental stage. Moreover, some researchers have found increased GFAP expression associated to gliosis after chronic (moderate) and acute low ethanol exposures, in mice[133,134]. These results indicate a high risk of neurodevelopmental disease in acute PAE or heavy drinkers. Conversely, no changes were observed in GFAP expression after low chronic ethanol exposure[133]. S100β is a classical biomarker for astrocytes, as the expression levels of S100β in these glial cells is very high. During neurite outgrowth, S100β is also secreted by proliferating astrocytes from cortical neurons. The accumulation of this protein in mature glial cells is associated with the microtubule network and neurotrophic activity[135]. Reduced levels of S100β were reported in mice after ethanol exposure[136], indicating a depletion in the number of proliferating astrocytes and impairment in the differentiation processes. Otherwise, Sox2 and Oct4 transcription factors regulate the embryonic stem cell pluripotency and the fate of cell lineages by a narrow dose-effect range[137]. Excess

of Oct4 compared to Sox2 leads cells to mesoendoderm differentiation, while the other way round, i.e., higher levels of Sox2, promotes neuroectoderm formation. Ethanol exposure of embryonic stem cells in early differentiation generates imbalances between Oct4 and Sox2, which modifies the cellular fate from neuroectoderm to mesoendoderm, altering the formation of the ectoderm lineage and its derived progenitors. The Oct4/Sox2 imbalance is considered one of the leading causes of developmental delay and anatomical disabilities of the CNS observed in FAS phenotypes[137].

1.3.4. Synaptogenesis

The developmental process of synaptogenesis involves biochemical and morphological changes in pre- and postsynaptic components. In rodents, maturation of synaptic connections occurs during the postnatal period (Figure 1)[138] and depends on the physicochemical compatibility of pre- and postsynaptic components and the exclusion of inadequate connections. Less harmful effects of alcohol exposure on synaptogenesis have been observed when administered after birth[60,61], although during neuronal development ethanol seriously alters some mechanisms related to synaptogenesis[60,61]. In a study using a rat model in which individuals were exposed to ethanol four weeks before and during pregnancy, the ultrastructural analysis of the cerebellum at PND 7 showed a delayed synaptogenesis and immature appearance of the presynaptic grid[61]. PAE affects the expression levels of synaptic proteins such as synapsin 1 and of other proteins of the presynaptic (GAP-43, synaptophysin, synaptotagmin) or postsynaptic machinery (MAP 2 and neurogranin). Moreover, ethanol interferes with the function of adhesion molecules such as NCAM (in a chick embryo model)[139] and L1 (in a mouse model)[140] involved in cell-cell interactions. During the neural processes of migration and morphogenesis, both proteins are involved in the organization and function of synaptic networks, which determine neuronal plasticity. Several studies in animal models (zebrafish) and cell cultures show decreased levels of NCAM after ethanol exposure[141,142]. In other studies, different patterns of NCAM expression were detected according to the developmental stage on which PAE occurs[139] or the NCAM isoform analyzed. For example, the highly sialylated form of NCAM is overexpressed after ethanol exposure but the NCAM 180 and NCAM 140 isoforms appear down-regulated in a rat model[143]. Other studies in animal models (mice and rats) have shown down-regulation of L1 following ethanol exposure[140,144].

1.3.5. Gliogenesis and myelination

Glial cells provide nutrients and physical support to neurons and regulate the presence of different proteins and components in the extracellular fluid surrounding neurons and synapses in the brain. They are essential for the normal development and

function of the CNS[145]. Neuroblast migration occurs through a scaffold provided by radial glia[146]. Microglia have macrophage functions and astrocytes preserve the ionic and trophic balance of the extracellular medium[147]. Oligodendrocytes synthesize myelin, therefore, this cell lineage preserves the myelin sheath and provides trophic support[148]. Schwann cells and oligodendrocytes are in charge of the isolation and myelination of neuronal axons[149]. Oligodendrocyte progenitor cells proliferate and differentiate into mature oligodendrocytes capable of myelinogenesis[150]. Thus, myelination begins later in neurodevelopment than in other processes, such as proliferation and migration, and progresses throughout adolescence in rodents[151,152]. The development of these cell lineages occurs in parallel to neurogenesis in several areas of the CNS[147]. These lineages are characterized by distinct developmental stages and sequential expression of different developmental biomarkers such as the nerve growth factor (NGF), neurotrophins (NT-3 and NT-4), the brain derived neurotrophic factor (BDNF), and the IGF-1 and IGF-2 factors. The BDNF is one of the most studied neurotrophins. Alcohol alters the levels of BDNF and its receptor tyrosine kinase B (TrkB). PAE induces a reduction of BDNF levels in the cortex and the hippocampus in rats at PND 7-8[153]. Some studies in rats show that TrkB levels decrease in specific brain regions, e.g., the hippocampus[153,154] and increase in the cortex[154]. The BDNF and its receptor are targets for ethanol damage. Consequently, imbalances between them may contribute to the development of FASD-like phenotypes, even in cases in which the levels of either compound remain unaltered. In general, the up-regulation of these neurotrophic factors show protective effects during development, promoting myelination, cell survival, and neural regeneration in pathological conditions[155].

Lancaster *et al*. showed that PAE reduces myelinogenesis and its persistence after birth in a rat model[62]. Severe gliosis and reduction of proteins related to myelin integrity (myelin-associated glycoprotein, myelin basic protein, myelin proteolipid protein, and myelin regulatory factor) were observed in male adult mice exposed to a binge (acute) pattern of PAE during gestation and lactation. This was followed by behavioral alterations in executive function and motor coordination[85]. These changes could be associated to the behavioral disabilities observed in FASD individuals. It has also been shown that exposure to alcohol activates toll-like receptor 4 signaling pathways (MAPK, NFκB) in a mouse model, leading to increased expression of pro-inflammatory mediators (COX-2, iNOS, HMGB1) and cytokines. Inflammation processes cause imbalances in myelinogenesis, impairments in synaptic links, and activation of the cell death mechanism[156].

1.3.6. Trophic support

CNS remodeling is a continuous process that not only takes place during development, but also throughout adulthood in response to environmental influences or genetically programmed events. Alcohol alters synaptic plasticity and neural function[157]. Several proteins used as biomarkers participate in neural plasticity processes. Histone deacetylase 2 alters the GluN2A/GluN2B balance (the major subunits of functional N-methyl-D-aspartate (NMDA) receptors) through changes in GluN2B expression, which leads to memory-impairing effects[158]. The neurotrophin family of proteins includes NGF, BDNF, NT-3, NT-4/5, and NT-6. It is well known that NGF and BDNF play important roles in PAE- and FASD-related pathogenesis. Various studies have shown that PAE disrupts neurotrophin pathways, thus affecting the organogenesis and development of brain structures in rodents[159,160]. NGF and BDNF exert their biological effects by activating some members of the tropomyosin-related kinase (Trk) family. NGF activates TrkA and BDNF binds to TrkB[161]. Stressful events, neurological injuries, or neuroendocrine alterations in rats increase blood levels of NGF[160]. Thus, NGF expression and the functional activity of NGF-target cells in the CNS are seriously affected by alcohol consumption. BDNF regulates neural cell survival and differentiation, as well as several functions related to neural plasticity such as learning and memory[162]. A recent study found that BDNF levels in the prefrontal cortex were significantly lower in the group of mice treated with ethanol in comparison to the control group[163]. The study concluded that the impairment in learning and memory observed in mice exposed to ethanol was associated to changes in BDNF levels. Stragier *et al*. showed that chronic and moderate alcohol consumption in C57BL/6J mice promotes a chromatin-remodeling process, leading to up-regulation of BDNF signaling. The authors suggest that this epigenetic regulation is an adaptive process to balance alcohol-induced cognitive disorders[164]. Another study in mouse reports a reduction of ethanol dependence after BDNF infusion in the prefrontal cortex[165], evidencing that BDNF levels in specific brain areas play a role in alcohol dependence. Boehme *et al*. studied the changes produced by voluntary exercise in hippocampal BDNF levels. Ethanol was delivered by intragastric gavage during the three trimester equivalents and individuals had free access to voluntary exercise on a running wheel during adulthood. Results showed increased BDNF levels in young adult females after voluntary exercise[46]. Recent studies suggest that matrix metalloproteinase-9 (MMP-9), a Zn^{2+} dependent extracellular endopeptidase, participates in neuronal plasticity, specifically in memory and learning[166,167]. Acute and chronic ethanol exposure up-regulates MMP-9 levels in the brain, particularly in the medial prefrontal cortex and hippocampus, in rats[168]. The vascular endothelial growth

factor (VEGF) is involved in the activity, plasticity, and survival of microvessels. Mice prenatally exposed to alcohol have reduced cortical vascular density, affected microvascular structure, and altered expression of VEGF and its receptor. VEGF may prevent microvessel plasticity disorders and death. As a mouse model shows, PAE exerts deleterious effects on the microvascular network, which suggests that vascular defects contribute to alcohol-induced brain injury[169]. *In vitro* studies show that ethanol also alters the expression and function of IGF-1 and IGF-2, leading to birth defects such as low head circumference at birth and microcephaly. These insulin-like growth factors are used by the organism as a general signal of cell survival, so that reduced IGF-I or IGF-II signaling by PAE in neurons activates cell death mechanisms by apoptosis or necrosis[170]. Other biomarkers such as DYRK1A act as general inhibitors of neural plasticity. Their over-expression in different brain areas due to environmental insults or stress situations, reduces neural plasticity in neurons promoting cognitive problems and intellectual disability[171–173]. Recent studies have demonstrated that some DYRK1A inhibitors such as the antioxidant epigallocatechin gallate (EGCG) improve long-term outcomes related with memory and executive function in individuals with Down's syndrome[171–173]. Although it is currently under study, the inhibition of DYRK1A might improve cognitive performance in pathologies associated to the loss of neuronal functions and plasticity, e.g., FASD, autism, or Down's Syndrome[174]. Furthermore, EGCG increases NGF expression by downregulating MMP-9. These proteins have been associated with FASD alterations during neurodevelopment[175].

1.3.7. Synaptic plasticity

Synaptic plasticity is the process through which long-term changes in synaptic communication occur[176].

Fontaine *et al*. studied the effect of prenatal exposure to ethanol in a rat model during the two trimester equivalent and PND 21 to 28 on long-term potentiation and depression, and depotentiation in the medial perforant path input to the hippocampus DG. Impairment of long-term potentiation was seen in both males and females, while long-term depression was only observed in males. These results suggest that PAE causes gender-impairment in synaptic plasticity in long-term depression[177]. Wong *et al*. focused their study on the contribution of microglia in synaptic plasticity. Using a third trimester equivalent mice model, ethanol was injected simulating a binge-drinking pattern. The authors found a deficit in experience-dependent synaptic plasticity in the visual cortex with no correlation to microglial function[178]. Shivakumar *et al*. administered ethanol to mice at PND 7 and showed that ethanol exposure produces epigenetic

changes that inhibit the activation of several synaptic plasticity genes. Combination with trichostatin A prevents learning and memory disorders in adult mice[179].

PAE negatively affects synaptic plasticity. Epigenetic changes, as well as damage to the microglia, may partially explain synaptic plasticity disorders in FASD models.

1.3.8. Apoptosis

Apoptosis is a critical pathway in fetal neurodevelopment. Programmed cell death systematically removes a large number of neural precursors from embryonic structures formed during development. PAE activates and deregulates cell death mechanisms, leading to the loss of cell lineages in the hippocampus, basal ganglia, or cerebellum and disappearance of critical structures in the brain such as the *corpus callosum*[63,64]. Activation of apoptosis is produced by an increase of reactive oxygen species (ROS) generated in ethanol metabolism (see section 4.1). ROS activate intrinsic and extrinsic apoptotic pathways, reducing the expression and function of the anti-apoptotic proteins Bcl-xL and Bcl-2 in a rat model[180]. A study using a mouse model shows that the function of the pro-apoptotic effectors Bak and Bax is directly affected by alcohol due to alterations in mitochondrial membrane fluidity and mitochondrial respiration dysfunctions, leading to the activation of the caspase cascade and subsequent generation of the active form of the effector caspase 3[181]. Consequently, some researchers have developed mitochondrial protective strategies to prevent alcohol-induced damage. Certain molecules, e.g., nicotinamide[182], stabilize mitochondrial membranes while others, e.g., antioxidants, prevent mitochondrial dysfunction induced by the production of ROS following ethanol exposure, in mouse. In addition, ethanol activates specific cell death pathways. More specifically, ethanol induces c-jun N-terminal-kinase phosphorylation, a mitogen-activated protein kinase associated with apoptosis, and GDNF may interfere with the activation of the c-jun N-terminal-kinase molecular pathway to prevent ethanol-induced apoptosis. Unlike other neurotoxic substances, ethanol does not interfere with the phosphorylation of extracellular signal-regulated kinases involved in the regulation of cell survival[183].

1.4. Diagnosis of fetal alcohol spectrum disorders

PAE results in a wide range of phenotypic manifestations and behavioral deficits in the offspring as described below.

1.4.1. Craniofacial anomalies

The key facial features used for a clinical diagnosis of FAS in humans include short palpebral fissures, thin upper lip vermilion, and smooth philtrum[184]. Based on human studies[218,219], in 2009 Fang *et al.* described a validated facial image analysis method for first time, based on a multi-angle image classification using micro-video images of mouse

embryos. This method, validated later by other researchers, allows discerning between embryos that have been exposed to ethanol from those who have not[185,186]. The use of a validated model in rodents is not only useful to study how PAE alters morphogenetic processes, but it also allow making associations between a change in a facial feature and the structure/function in the CNS.

Alcohol exposure during essential periods of embryonic development results in craniofacial dysmorphology (Figure 3). Several studies have used chick and murine PAE models to demonstrate the correlation between craniofacial anomalies, apoptosis induction, and altered migration of neural crest cells[187–189]. A series of facial anomalies may present in FASD associated to PAE during the premigratory period of neural crest cells (Figure 2). At this stage, ethanol induces calcium transients that activate CaMKII that mediates the loss of transcriptionally active β-catenin, triggering the apoptosis of populations of neural crest cells. Genetic factors play an important role in the vulnerability to alcohol-induced craniofacial dysmorphology. Sonic Hedgehog signaling, platelet-derived growth factor subunit A, Vang-like protein 2, or ribosomal biogenesis genes are of special relevance in neural crest development[190]. Studies using FASD-like phenotype rodent models, in which dose and timing of ethanol exposure is controlled, show structural alterations in the head and face[191,192] resembling the anomalies observed in humans under PAE situations[193].

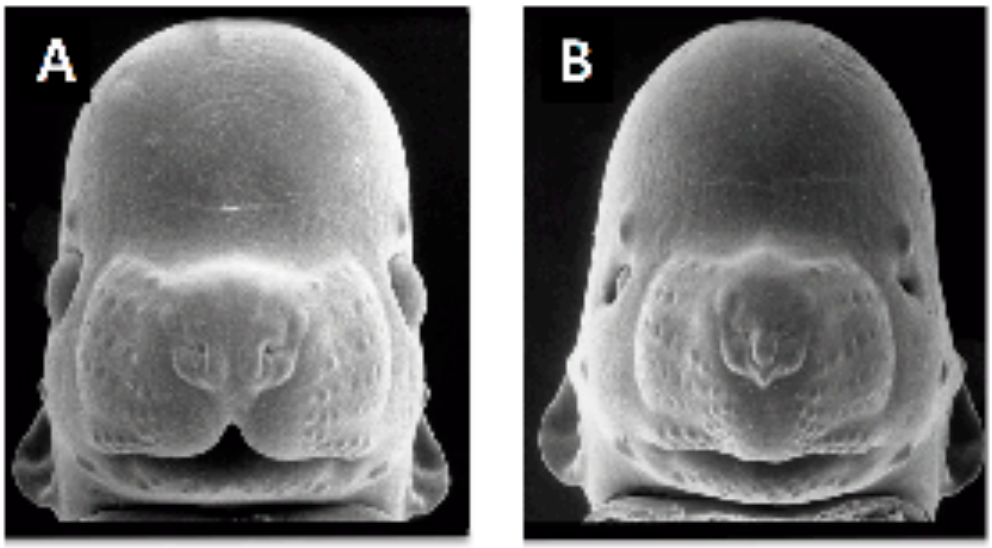

Figure 3. Facial dysmorphology induced by prenatal ethanol exposure. Representative examples of a control animal (A) and a fetus severely affected by ethanol exposure (B). Microcephaly, short palpebral fissures, thin upper lip vermillion, and smooth philtrum define the FAS-like phenotype (B). FAS-like facial features are induced in the mouse by maternal alcohol exposure on gestational day 7 and 8.5 (equivalent to the third gestational week in humans). Courtesy of Prof. Kathie Sulik, University of North Carolina-Chapel Hill (271) (https://www.teratology.org/primer/fas.asp).

Several studies have examined the craniofacial anomalies in FASD-like rodent models. According to Godin *et al.*, intraperitoneal administration of two injections of ethanol at 2.9 g/kg in mice on GD 7 (equivalent to post-fertilization week 3 in humans), causes a series of facial dysmorphologies similar to those seen in FAS children. These defects include median facial cleft, cleft palate, micrognathia, pituitary agenesis, and third ventricular dilatation and heterotopias[44]. However, intraperitoneal alcohol exposure of two 25% dosages of ethanol at 2.9 g/kg delayed to GD 8.5 in mice produces a different pattern of dysmorphologies, such as shortening of the palpebral fissures, mild hypoplasia, and shortened upper lip, but a preserved philtrum[69]. Variations in FAS-like facial phenotypes depend on exposure timing, implying different facial features when considering this variable (Figure 2). New techniques for FASD diagnosis include methods to identify potentially at-risk individuals based on the identification of subtle and subclinical facial characteristics[194]. A computerized system for detecting facial characteristics using three-dimensional facial imaging and computer-based dense-surface modelling has been developed[194,195]. This approach has been compared against standard dysmorphology physical examination for FAS diagnosis showing high similarities[196]. More recently, a new technique based on 3D MicroCT scan performed on pups prenatally exposed to alcohol has been created[197]. This method shows that craniofacial bones might be reliable and sensitive indicators of PAE in mouse pups exposed to 4.2% alcohol v/v for two weeks before pregnancy and GD 7-16. The same study confirmed that the neurocranium (cranial skeleton) is more sensitive to alcohol than the viscerocranium (facial skeleton). Other researchers characterized concurrent face-brain phenotypes in mouse fetuses exposed to two 25% intraperitoneal dosages of ethanol at 2.9 g/kg on GD 7 or GD 8.5 using MRI imaging and dense surface modeling-based shape analysis[69]. Differences in facial phenotype linked to ethanol exposure GD were found, being more subtle when the exposure was on GD 8.5. Both phenotypes were associated with unique volumetric and shape abnormalities of the septal region, pituitary, and olfactory bulbs. These findings justify the need to improve the current diagnostic criteria to better capture the full range of facial and brain dysmorphology in FASD.

1.4.2. Brain and neurobehavioral deficits

Brain organogenesis is the most severely affected process by alcohol exposure[198] and there is a general consensus in relation to the effects of PAE on the hippocampus, cerebellum, and the *corpus callosum*[199,200]. Important asymmetry of the hippocampus is observed in FAS children, with the left lobe being smaller than the right lobe[8]. The

cerebellum, associated with balance, coordination, and learning capacity, and the anterior part of the vermis develop hypoplasia when exposed to ethanol[7].

The *corpus callosum* is particularly vulnerable to ethanol exposure and, in some cases, may lead to total (agenesis) or partial (hypoplasia) loss of structure[201]. The most affected areas of the *corpus callosum* are the front (genu) and back (splenium and isthmus), appearing smaller and displaced from their usual spatial location in the brain[6]. Basal ganglia is responsible for motor and cognitive abilities, presenting a smaller size in patients with FAS, particularly the area of the caudal nucleus associated to cognitive abilities such as spatial capacity[202]. A recent study using 3D surface MRI techniques showed abnormalities in the cortical folding (gyrification) in FASD children. These findings are directly correlated with the intelligence quotient (IQ)[203]. Future research with MRI techniques to evaluate rodent gyrification may prove to be useful to further understand the relationship between cortical development and cognitive disorders in humans.

Broadly, timing of ethanol exposure[204] has a clear impact on the CNS and elicits specific brain and behavioral deficits and disorders in motor and cognitive functions[205] (Figure 2).

Different standardized tests in rodents have been used to assess FASD-related abnormalities. The hippocampus is one of the most damaged structures with ethanol exposure, for which reason many studies assess hippocampal function. Spatial learning is commonly evaluated to demonstrate hippocampal disorders[47,85,206]. Different authors describe long-term motor coordination impairments learning and memory deficiencies in adult male mice prenatally exposed to alcohol[85], behavioral effects in rats following short-term PAE[207], or depressive-like behaviors in adult rats exposed to ethanol throughout the three-trimester equivalents[208]. Understanding the characteristics of behavioral disorders in mice is necessary to identify the effects of PAE in these models.

1.4.3. Fetal growth restriction

Interference of ethanol on maternal nutrition may differ. Being a source of energy, alcohol blocks the absorption of other nutrients, including proteins, and hinders intestinal transport of essential nutrients. Due to its effects on the liver, alcohol causes changes in metabolic and nutrient utilization. PAE leads to maternal nutritional deficiencies that result in fetal growth impairments[37].

PAE also impairs placental angiogenesis[209] leading to fetal growth restriction (FGR)[16]. The growth curves defined by Dilworth *et al*. are a useful tool to define the frequency distribution of mouse weight. Any fetus with a weight below the fifth centile was considered growth restricted[76]. Middaugh *et al*. characterized the impaired growth of C57BL/6 mice prenatally exposed to alcohol[210] and showed the effect of alcohol on

fetal growth when administered in the second and third trimester equivalents[210]. Tran et al. used a FAS-like rat model that equalizes BAC peak in the different developmental periods. Pups prenatally exposed to ethanol for all trimesters equivalent or for the second trimester equivalent showed significantly lower body weights[211]. The latter stages of pregnancy, rather than early pregnancy, appear to be more sensitive to PAE in terms of postnatal growth. Thus, the growth deficit produced by PAE depends on the timing of the insult during gestation, which will affect certain neural or neuroendocrine systems at that stage of development.

Other authors have described the effects of ethanol on trophoblasts and placental permeability. Alcohol exposed placentas showed altered branching morphogenesis in the labyrinthine zone and suppression of invasive trophoblastic precursors[77]. Permeability was also affected by the altered structures in the barriers that separate feto-maternal blood circulation[212]. This altered process compromised fetal growth and placentation in a dose-response manner[77]. The vascular endothelial growth factor (VEGF), a regulator of angiogenesis and permeability inducer, was up-regulated in mouse placentas after occasional acute alcohol exposure[212]. On the other hand, continuous exposure throughout the perigestational period up to early organogenesis produces a reduction of VEGF expression and up-regulation of the vascular endothelial growth factor receptor (VEGF-R), a negative regulator of embryogenic angiogenesis[213]. These alterations contribute to a deficient decidual angiogenesis. Finally, the placental growth factor (PLGF), a member of the VEGF family, also involved in angiogenesis and vasculogenesis during the embryonic period, is regulated in a different manner depending on the developmental stage[212]. In addition, insulin-like growth factors (IGFs), especially IGF-II, are critical regulators of fetal growth and placentation. Although IGF-II levels were found to be increased in placentas of a FASD-like rat model, its bioavailability is reduced as a result of IGF receptor overexpression, which contributes to disorders in placentation and FGR[214]. PAE produces an increase of placental Bax and Bak pro-apoptotic proteins and a decrease of the anti-apoptotic Bcl-e protein, promoting fetal loss[215]. Therefore, altered growth factors in conjunction with malformations of the placental barrier may contribute to placental malfunction and permeability alterations in the feto-maternal barrier.

1.5. Alcohol-induced oxidative stress

Multiple pathological effects derived from alcohol exposure during fetal development have been described, which depend on the studied organ, region and cell type, and the stage of pregnancy at which the fetus is exposed to ethanol[216]. Teratogenic effects of PAE include oxidative stress damage and other pathophysiology mechanisms, e.g.,

dysregulation of the neuroimmune system, neurotransmitter disorders, and epigenetic modifications.

Free radicals are highly reactive molecules, many of which contain oxygen. They are able to produce oxidative stress, subsequent damage to cellular components, including membranes, proteins and DNA, and can induce apoptosis. There is evidence supporting a role for oxidative stress in alcohol teratogenesis[217].

Many pathways may contribute to the ability of alcohol to induce a state of oxidative stress. Ethanol is metabolized in the liver of adult individuals via the alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) families of enzymes (Figure 4), leading to moderate ROS production, e.g., hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH-). ROS are eliminated by endogenous antioxidant mechanisms directed by catalase, superoxide dismutase (SOD), and the antioxidant molecule glutathione (GSH)[218]. After high alcohol intake, the catalytic activity of ADH and ALDH becomes saturated and an alternative pathway mediated by the cytochrome P450 2E1 enzyme is up-regulated to metabolize ethanol to acetaldehyde, producing high amounts of ROS. ROS-sensing transcription factors, such as the nuclear erythroid 2-related factor 2 (Nrf2), activate the oxidative stress response mechanisms when moderate levels of alcohol-derived ROS are present, up-regulating antioxidant enzymes and proteins involved in DNA repair. Imbalances between ROS-producing pathways (following PAE) versus the endogenous antioxidant and DNA repair mechanisms promote down-regulation of the detoxification pathways[218,219]. NADPH oxidase (NOX) also plays an important role in alcohol teratogenicity. Alcohol exposure induces NOX enzyme activity and increases mRNA expression of the NOX regulatory subunits. NOX inhibitors, e.g., diphenyleneiodonium, have shown to be protective, diminishing the generation of alcohol-induced ROS, caspase-3 activity, and apoptosis[220]. The decrease of the antioxidant system affects specific regions of the CNS such as the cerebellum, hippocampus and cortex, as well as the placenta[221,222]. The fetal brain is particularly sensitive to PAE because the ADH isoform expressed in this tissue during development is a class II isoenzyme (ADH4). This isoform is less efficient for alcohol catabolism than other isoforms expressed in adults[223]. The mechanisms involved in antioxidant response are physiologically downregulated during development[224–227], increasing brain´s vulnerability to ethanol. The excess of ethanol also activates the lactate pathway in fetal liver, generating a deficit of glucose in the bloodstream that affects nervous tissues especially[228]. Imbalances of ROS activate the mechanisms of inflammation[229] mediated by cytokines such as IL-6 or the NLRP3 inflammasome, a multi-protein intracellular complex responsible for processing and secretion of the pro-inflammatory cytokines IL-1β and IL-18[230].

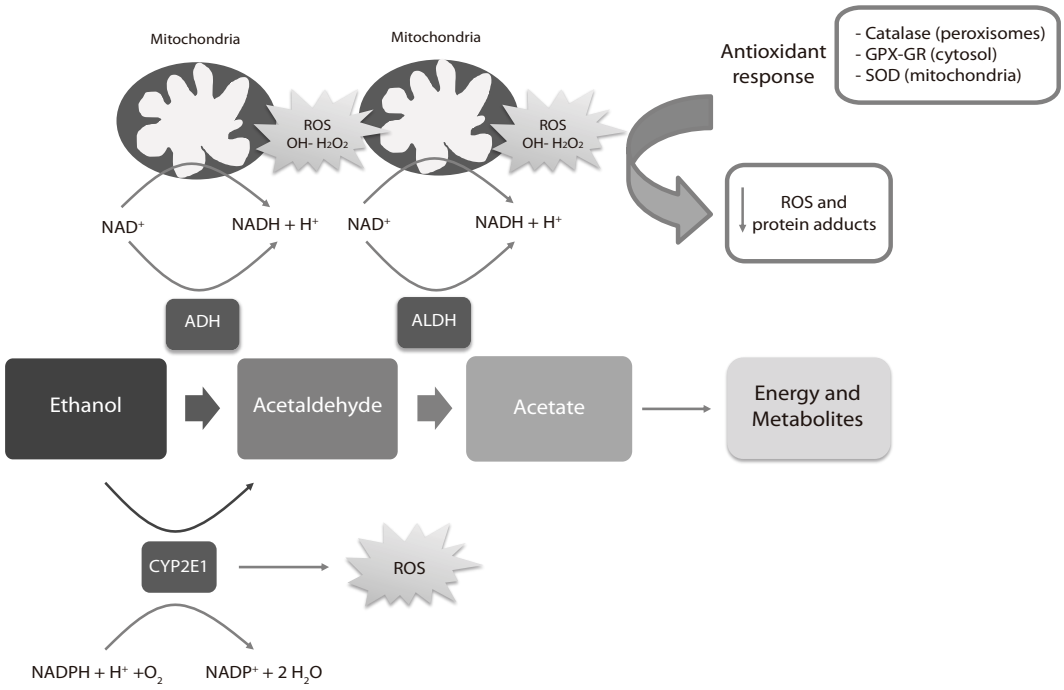

Figure 4. Alcohol metabolic pathway in the liver. ADH: alcohol dehydrogenase. ALDH: aldehyde dehydrogenase. ROS: reactive oxygen species. SOD: Superoxide Dismutase, GPX: Glutathione peroxidase. GR: Glutathione reductase. The ADH, ALDH enzymes and the P450 2E1 cytochrome participate in the oxidative metabolism of alcohol. The activity of the P450 2E1 cytochrome in alcohol metabolism and re-oxidation of NADH via the electron transport chain in the mitochondria results in the formation of ROS. Catalase in peroxisomes, SOD in mitochondria and GPX-GR in cytosol are activated by an increase of oxidative stress to reduce the levels of ROS. High amounts of ROS lead to a down-regulation (negative feedback) of the antioxidant response. Own authorship.

Tissue homeostasis is also affected by ROS[231], which cause changes in critical cell functions as signal transduction related to the metabolism of macromolecules (lipids, proteins, RNA, and DNA)[229]. E.g., ROS promote the modification of 8-oxoguanine in DNA during embryogenesis[232,233], corrected by the enzyme oxoguanine glycosylase 1[234]. Calcium homeostasis and protein folding, modification, and secretion in the endoplasmic reticulum are also altered by ROS, as well as mitochondrial respiration, affecting its morphology and function. Moreover, activation of autophagy, programmed (apoptosis) and non-programmed (necrosis) cell death are promoted by oxidative stress[231,235,236].

Some studies have assessed the long-term consequences of PAE on oxidative stress and the intracellular redox state. Dembele *et al*. found an relation between continuous administration of PAE with increased levels of oxidative stress in adult rats (PND 90), characterized by high levels of protein carbonyls, lipid peroxides, high expression of SOD, and low levels of GSH[237]. Similar results have been reported by other authors, who showed an association between chronic PAE at different concentrations

with increased levels of distinct oxidative stress and lipid peroxidation markers in adolescent and adult rodents[238,239]. Chu *et al*. found a correlation between PAE and apoptotic (p53) and DNA oxidation markers (8-hydroxydeoxyguanosine) in adult rats[239]. Brocardo *et al*. reported depressive and anxiety-like behaviors and high levels of lipid and protein peroxidation in adult rats (PND60) who were given ethanol throughout the three-trimester equivalents[208]. Their findings also indicate an association between voluntary exercise, which increased the endogenous antioxidant pathways in brain, and reduced oxidative stress and depressive/anxiety-like behaviors. Similarly, binge PAE (GD 17–18) increased the levels of lipid peroxidation and oxidative stress, apoptotic activation via caspase-3 activity, and DNA fragmentation, decreasing antioxidant molecules as GSH[240,241]. Alcohol exposure induces the activity of the NADPH oxidase (NOX) and increases mRNA expression of NOX regulatory subunits.

1.6. Antioxidant strategy in fetal alcohol spectrum disorders

There are no specific treatments for FASD. Alcohol abstinence during pregnancy is the only effective recommendation against FASD. The physical defects and mental disorders typically persist for a lifetime. However, early interventions, e.g., nutritional strategies and environmental enrichment, may help reduce some FASD effects and prevent some secondary disabilities. Although the underlying mechanisms of ethanol neurotoxicity are not completely determined, the induction of oxidative stress is considered one of the central processes linked to the development of this disease[242]. Different strategies based on treatments with nutritional supplements such as natural antioxidants are currently under study to determine their role in reducing PAE-related oxidative stress damage.

1.6.1. Endogenous and exogenous antioxidant mechanisms

An antioxidant is a molecule capable of slowing down or preventing the oxidation of other molecules. Oxidation is a chemical reaction of electron transfer from a substance to an oxidizing agent. Oxidation reactions can produce radicals that are able to damage cells. Antioxidants prevent these reactions by removing intermediates from the radical and inhibiting other oxidation reactions by oxidizing themselves. Oxidative stress is promoted by low levels of antioxidants or inhibition of antioxidant enzymes, which causes severe damage in the cells and/or leads to cell death.

Knowledge on the mode of action of antioxidants may support their use to reduce the teratogenic effects of alcohol. Antioxidants can be divided into three large groups:

- Antioxidant enzymes that catalyze ROS degradation. SOD, peroxidases, and the enzyme catalase are antioxidant enzymes.
- Antioxidants that prevent chain reactions. When a free radical interacts with

another molecule, secondary radicals are generated that in turn can react with other targets so that chain reactions are triggered with other targets producing even more radical species. Antioxidants interrupt these chain reactions because they receive or give electrons to a radical, forming a stable intermediate compound. As a result of the reaction, the antioxidant oxidizes and must be regenerated or replaced in order to become active again. Some examples of antioxidants are tocopherols, flavonoids, carotenoids, and vitamin C.

- Transition metal binding proteins sequester iron and copper ions so that they are not available to react with radicals such as SOD and hydrogen peroxide. This prevents the formation of the highly reactive hydroxyl radical. Some examples of this type of proteins are transferrin, ferritin, lactoferrin, and ceruloplasmin.

Oxidative stress has been associated with the pathogenesis of many human diseases. For this reason, there is special interest in the use of antioxidants for the treatment or prevention of various pathologies, e.g., neurodegenerative diseases[243].

1.6.2. Natural antioxidants: green tea

Tea is the second most consumed drink in the world, after water[244]. It is extracted from the leaves of *Camellia sinensis* and mainly produced in four varieties, white, green, oolong, and black, depending on the level of oxidation and fermentation[245]. Daily intake of green tea provides several health benefits, e.g., anti-inflammatory, anti-cancer, antimicrobial, and antioxidant, helping reduce the risk of developing certain diseases[246]. The health benefits of green tea are mainly attributed to its antioxidant properties[247]. For that reason, green tea extracts have been evaluated in diseases associated with the increase of ROS and oxidative stress, such as cancer and cardiovascular diseases[248,249]. Moreover, other molecular mechanisms such as signaling pathways, modulation of the activity of certain enzymes, and several interactions with membrane receptors related to cognitive functioning have also been described for green tea components in the treatment of neurodegenerative pathologies as Alzheimer's disease[243,250].

Most of the health-promoting effects of green tea are associated to its polyphenol content[251], particularly flavonoids. The main flavonoids in green tea, catechins, make up to 30%–40% of its solid components. The major catechins in green tea are epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG).

1.6.3. Epigallocatechin gallate

EGCG (Figure 5), the most abundant flavonoid, represents approximately 59% of total catechins in green tea[246]. Many of the beneficial properties of green tea are

attributed to this compound, so that EGCG has recently been put forward as a potential therapeutic agent[252,253]. Some of the health effects are related to the antiproliferative activity of EGCG, as it interferes with intracellular signaling cascades inhibiting cell growth at the G1 phase, triggering apoptosis. In this framework, EGCG has been proposed as a cancer chemopreventive compound[254,255]. Other beneficial properties of EGCG have been described: its metabolic effects in reducing the risk of type 2 diabetes and associated cardiovascular complications[256]; its antimicrobial activity by preventing bacteria to bind to their host cells; catechins bind to the lipid bilayer and alter the bacterial cell membrane; its role in preventing and reducing viral infections. EGCG also exhibits a protective role in some neurodegenerative diseases, e.g., Alzheimer´s disease[246] or after a neural injury[257]. Additionally, a recent research has demonstrated that this flavonoid improves cognitive performance and adaptive functionality in individuals with Down's syndrome by modulating the overexpression of the dual specificity tyrosine phosphorylation regulated kinase 1A (Dyrk1A)[171]. This protein is encoded by the DYRK1A gene, involved in signaling pathways that regulate cell proliferation, neural plasticity, and neurogenesis[258].

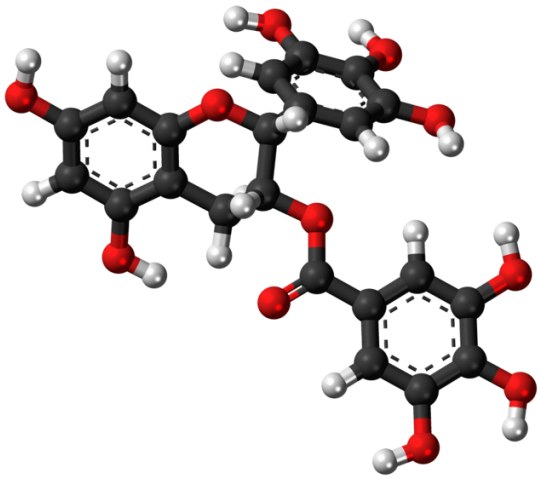

Figure 5. Molecule model of epigallocatechin gallate. Colour code: carbon (C), black; hydrogen (H), white; Oxygen (O), red. Creative Commons license.

1.6.3.1. Metabolism and bioavailability

The eight free hydroxyl groups of the bioactive EGCG compound, favor its biological functions[259]. Like other phenolic compounds, EGCG shows low bioavailability, 0.1-0.3% in humans[260,261]. EGCG availability depends on the absorption, distribution, metabolism, and excretion processes, reaching peak plasma concentration after one to five hours[262] and being undetectable 24 hours after oral dosing[263]. The acidic pH

environment in the stomach favors the structural stability of EGCG[264]. In its transit through the duodenum, EGCG goes through the digestion process mediated by the pancreas and neutralization by the secretion of bile salts[263,265]. Next, a fraction of EGCG is absorbed by the enterocytes in the small intestine. Low concentrations of EGCG reach peripheral blood because of the passage from the small to the large intestine. Once in the intestine, EGCG is catabolized by local microbiota through deconjugation and degradation processes[259]. Gut microbiota promotes up to eleven catechin products by ring fission in different species, present in free and conjugated forms in the plasma. *In vitro* data suggest that EGCG forms cross the blood-brain barrier and reach the brain parenchyma. Once distributed throughout the brain, EGCG promotes neuritogenesis, showing an important role in suppressing neurodegenerative diseases[266]. Finally, liver cells metabolize the remaining EGCG, transforming methylated, sulfate, and glucuronide intermediates that will be excreted in urine[267].

Moreover, stability of EGCG is affected by various factors, with notable differences in pharmacokinetic parameters among individuals[256,268]; for example, alkaline pH and high temperatures affect its structural stability, promoting degradation and thus reducing absorption[269]. Studies on EGCG bioavailability in humans show contradictory results. Some reports conclude that the intake of EGCG in combination with specific nutrients, such as omega-3 fatty acids[270], vitamins as ascorbic acid[271], and minerals as selenium or chrome, improve the bioavailability of EGCG enhancing its antioxidant properties. Conversely, recent studies conclude that EGCG oral bioavailability in humans is low when administered with food[263,272].

The hydrophilic-lipophilic balance of EGCG restricts its bioefficiency in lipophilic environments affecting its conformation and effectiveness. Modifying its structure through esterification with aliphatic molecules, such as long-chain fatty acids, may improve absorption and boost its bioavailability[273]. Moreover, these ester derivatives have shown higher antiviral and antioxidant properties than the non-esterified EGCG molecule[273–275] and more effective neuroprotection than non-modified EGCG in cellular models of neurological diseases[276]. Thus, EGCG esters may be used in nutrition as lipophilic alternatives without hampering its beneficial properties.

1.6.3.2. Antioxidant activity

EGCG is a powerful antioxidant. Under pathological conditions with increased oxidative stress (e.g., ischemia/reperfusion, alcoholic fatty liver, or obesity-induced inflammation), EGCG supplementation may provide protection against cell damage caused by free radicals.

Green tea polyphenols scavenge ROS by generating more stable phenolic radicals. The B and D rings of EGCG have a galloyl group. In vitro studies show how EGCG reacts with O2-, leading to oxidation of the D ring; thus, EGCG scavenges OH and O2-[277]. Furthermore, some studies show positive correlations between the phenolic content in green tea and antioxidant activity, measured by ferric reducing/antioxidant power and oxygen radical absorbance capacity assays[278,279]. EGCG inhibits the increase of H_2O_2 and MDA in murine models[280], arranges its phenolic groups to contribute to its antioxidant activity[281], protects against ROS-mediate DNA damage[282], and potentiates the cellular defense capacity against oxidative stress via Nrf2-mediated synthesis of antioxidant enzymes such as hemeoxygenase-1, glutamate cysteine ligase, glutathione peroxidase, and glutathione S-transferase [283].

In addition, EGCG directly interacts with proteins and phospholipids in the plasma membrane regulating signal transduction pathways, transcription factors, DNA methylation, mitochondrial function, and autophagy to exert many of its beneficial biological actions[284]. These effects depended on cell type, stress conditions, and EGCG concentration[284].

1.6.3.3. Neuroprotective effect

Different studies show the benefits on cognitive function of diet plans that include tea[285]. Recent research suggests that EGCG in green tea may play a role in improving neurological cell function and preventing degenerative brain diseases as in Parkinson's disease, Alzheimer's disease, or Down's syndrome. The targets of green tea catechins include the abnormal accumulation of fibrous proteins (e.g., Aβ and α-synuclein), pro-inflammatory and pro-apoptotic proteins, as well as oxidative stress response transcription factors, which are associated (all of them) with neuronal cell dysfunction and cell death in the cerebral cortex[286].

Alzheimer's disease is a neurodegenerative disorder characterized by β-amyloid peptide (Aβ) accumulation and neurofibrillary tangle formation. Some studies have reported that tea consumption is inversely associated with the risk of developing Alzheimer´s disease[287]. The expression of the disease depends on the amount and distribution of Aβ. Aβ induces synaptic dysfunction, disrupts neural connectivity, and induces brain region-specific neuronal death[246]. Several studies show the beneficial effect of EGCG on soluble Aβ levels and Aβ-induced behavioral, memory, and coordination impairment in murine models [288,289].

On the other hand, Parkinson's disease is a neurodegenerative disease caused by the loss of dopamine-producing nerve cells. Several studies suggest that the intake of tea has a protective effect on this disorder[290]. Flavonoids may stimulate the

phosphorylation of the transcription factor cAMP-response element-binding protein, a regulator of neuronal viability and synaptic plasticity, and inhibit NADPH oxidase activity[291].

Finally, Down's syndrome is characterized by altered neuro-architecture, deficient synaptic plasticity, and excitation-inhibition imbalance in critical brain regions for learning and memory. A combined therapy with environmental enrichment and EGCG, improved cortico-hippocampal-dependent learning and memory. Possible mechanisms for cognitive improvement was a rescue of cornu ammonis 1 (CA1) dendritic spine density and normalization of the proportion of excitatory and inhibitory synaptic markers in CA1 and the DG[292]. In addition, overexpression of DYRK1A (an inhibitor of neural plasticity) has been found in individuals with Down's syndrome. Recent studies have demonstrated that some DYRK1A inhibitors, such as the antioxidant EGCG, improve long-term outcomes related with memory and executive function in this population[171–173]

EGCG has been shown to help prevent and treat neurodegenerative diseases and may be useful for the therapeutic management of Fetal Alcohol Syndrome neurological effects.

HYPOTHESIS

2. HYPOTHESIS

Prenatal alcohol exposure produces different degrees of fetal growth restriction, placental disorders, and brain impairments in humans depending on the type of exposure (acute or binge versus chronic moderate or Mediterranean). EGCG therapy administered under optimal pharmacokinetic conditions may attenuate these abnormalities.

The specific hypotheses of this doctoral thesis are:

1. Binge human-like pattern of PAE produces more severe fetal growth restriction and placental disorders than Mediterranean PAE
2. Binge human-like pattern of PAE produces more severe brain impairment in comparison to Mediterranean PAE
3. EGCG therapy administered under optimal pharmacokinetic conditions may mitigate placental and brain alterations produced by PAE according to the two human-like drinking patterns (binge versus Mediterranean)
4. Highest plasma concentrations are reached with oral EGCG administration without food supplements
5. Food supplements improve the stability of oral EGCG administration

OBJECTIVES

3. OBJECTIVES

The main objectives of this thesis are to evaluate the protective effect of EGCG on a FASD-like mouse model exposed to two human-like drinking patterns (acute or binge versus chronic moderate or Mediterranean) and assess the specific EGCG bioavailability profile in humans under different nutritional conditions.

The specific objectives of this doctoral thesis are:

1. To assess the effects of binge versus Mediterranean alcohol exposure on fetal growth and placenta, based on fetal and placental weight and placental angiogenesis biomarkers
2. To analyze the effects of binge versus Mediterranean alcohol exposure on maturation, differentiation, and plasticity in fetal brain processes based on specific neuronal biomarkers
3. To study the protective effect of EGCG on fetal growth, placental development, and neurogenesis processes
4. To analyze the bioavailability of oral EGCG administered alone or with different food supplements in healthy volunteers
5. To evaluate the pharmacokinetic parameters of EGCG in healthy volunteers

METHODS AND RESULTS

4. METHODS AND RESULTS

Article 1. Epigallocatechin gallate ameliorates the effects of prenatal alcohol exposure in a fetal alcohol spectrum disorder-like mouse model

In this publication, we evaluated the protective effect of EGCG on a FASD-like mouse model based on the two human-like drinking patterns (binge versus Mediterranean). The aims in this work were:

1. To assess the effects of binge versus chronic alcohol exposure on fetal growth and placenta, based on fetal and placental weight and placental angiogenesis biomarkers

2. To analyze the effects of binge versus chronic alcohol exposure on maturation, differentiation, and plasticity in fetal brain development based on specific neuronal biomarkers

3. To study the protective effect of EGCG on fetal growth, placental development, and neurogenesis processes

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Epigallocatechin Gallate Ameliorates the Effects of Prenatal Alcohol Exposure in a Fetal Alcohol Spectrum Disorder-Like Mouse Model

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Abstract: Fetal alcohol spectrum disorder is the main preventable cause of intellectual disability in the Western world. Although binge drinking is the most studied prenatal alcohol exposure pattern, other types of exposure, such as the Mediterranean, are common in specific geographic areas. In this study, we analyze the effects of prenatal alcohol exposure in binge and Mediterranean human drinking patterns on placenta and brain development in C57BL/6J mice. We also assess the impact of prenatal treatment with the epigallocatechin-3-gallate antioxidant in both groups. Study experimental groups for Mediterranean or binge patterns: (1) control; (2) ethanol; (3) ethanol + epigallocatechin-3-gallate. Brain and placental tissue were collected on gestational Day 19. The molecular pathways studied were fetal and placental growth, placental angiogenesis (VEGF-A, PLGF, VEGF-R), oxidative stress (Nrf2), and neurodevelopmental processes including maturation (NeuN, DCX), differentiation (GFAP) and neural plasticity (BDNF). Prenatal alcohol exposure resulted in fetal growth restriction and produced imbalances of placental angiogenic factors. Moreover, prenatal alcohol exposure increased oxidative stress and caused significant alterations in neuronal maturation and astrocyte differentiation. Epigallocatechin-3-gallate therapy ameliorated fetal growth restriction, attenuated alcohol-induced changes in placental angiogenic factors, and partially rescued neuronal nuclear antigen (NeuN), (doublecortin) DCX, and (glial fibrillary acidic protein) GFAP levels. Any alcohol consumption (Mediterranean or binge) during pregnancy may generate a fetal alcohol spectrum disorder phenotype and the consequences may be partially attenuated by a prenatal treatment with epigallocatechin-3-gallate.

Keywords: FASD-like mouse model; prenatal alcohol exposure (PAE); binge alcohol drinking pattern; moderate alcohol drinking pattern; neural plasticity; angiogenesis; neural maturation; neural differentiation; neurodevelopmental disorders; natural antioxidants; epigallocatechin-3-gallate (EGCG)

1. Introduction

Alcohol, the most commonly used teratogen, triggers many deleterious effects in the offspring when consumed during pregnancy, with the consequences falling under the umbrella of fetal alcohol spectrum disorder (FASD) [1], of which fetal alcohol syndrome (FAS) is the most severe form of prenatal alcohol exposure (PAE) impairment. FAS is characterized by craniofacial dysmorphology, growth retardation, and central nervous system dysfunctions [1].

Different factors contribute to the pathophysiology of FASD. The teratogenic effects of PAE have great impact on the placenta, the central regulator of the intrauterine environment [2]. FASD in vitro and animal model studies have shown the detrimental effects of PAE on placental size, structure, and function [3] and alterations in the methylation pattern of placental genes affecting its growth and on fetal development [4–6]. Moreover, ethanol alters the expression of several placental angiogenic factors, e.g., the vascular endothelial growth factor A (VEGF-A), its receptor VEGFR, and the placental growth factor (PLGF) [7]. Ethanol diffuses through the placenta and rapidly distributes into the fetal compartment, accumulating in the amniotic fluid [8]. The efficiency of the alcohol dehydrogenase isoforms and aldehyde dehydrogenases enzymes expressed by the fetus for alcohol metabolization is limited. Furthermore, CYP2E1 activity, the alternative pathway to metabolize alcohol, is upregulated during development, generating high levels of reactive oxygen species (ROS), which intensify the damage caused by alcohol. In addition, the fetal brain is particularly vulnerable to alcohol due to the downregulation of the antioxidant response in this tissue [9,10]. Thus, PAE affects the central nervous system in all stages of brain development (neurulation, proliferation, migration, differentiation, synaptogenesis, gliogenesis, myelination, apoptosis, and plasticity) through a variety of mechanisms that include oxidative stress and the direct alteration of the epigenetic pattern in the neural lineages [11,12]. Therefore, ethanol may alter the expression of a wide range of neural biomarkers, e.g., the neuronal nuclear antigen (NeuN) [13], doublecortin (DCX) [14], glial fibrillary acidic protein (GFAP) [15], and the brain-derived neurotrophic factor (BDNF) [16], as well as of the oxidative stress biomarkers, e.g., the nuclear factor erythroid 2-related factor 2 (Nrf2) [17].

There is a direct association between the dose and timing of PAE and FAS characteristics [18]. FAS phenotypes are reported in the offspring of mothers who consume high doses of alcohol (acute or binge models) during different stages of pregnancy [19]. Binge drinking is defined by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) as a drinking pattern that brings blood alcohol concentration (BAC) to 0.08 g/dL [20] (this typically occurs with four/five drinks in women/men, respectively, in about two hours) [20]. In Mediterranean countries, mild or moderate patterns of alcohol consumption (chronic model) are more frequently seen, with reduced prevalence of FAS compared to European Eastern countries. However, nearly one out of 14 children diagnosed with FASD were exposed to only one drink per day during their fetal development [21]. Moderate drinking is defined by the NIAAA as up to one/two drinks a day in women/men, respectively [20].

Animal models allow the control of variables linked to FAS phenotypes, e.g., dose, timing, and developmental stage of alcohol exposure during pregnancy. High BACs ($\geq$100 mg/dL), similar to the levels reached in humans [22,23], can be achieved with mouse models of alcohol exposure, to mimic the human binge-drinking pattern. The FAS-like phenotype is easily identified in the mouse offspring when using an alcohol binge-drinking pattern. However, the human moderate drinking pattern is not well established in mouse models, and causal relations between moderate PAE and FASD manifestations are difficult to determine due to the incomplete phenotype and the confounding factors [24].

To date, the evidence indicates there is no safe amount of alcohol consumption during gestation [25]. Furthermore, there is no treatment for FASD, no early intervention for the offspring, and no long-term follow-up to improve behavioral disabilities [26]. FASD research focuses on the underlying mechanisms of ethanol teratogenesis, such as the oxidative stress generated by alcohol metabolism [27]. Moreover, PAE epigenetic changes are the leading

cause of the alterations produced by PAE in the neurobiological system [28]. Potential therapies to reduce oxidative stress and epigenetic alterations include natural antioxidants such as epigallocatechin-3-gallate (EGCG), which has been used as a therapeutic tool in oxidative stress-related pathological processes such as cardiovascular diseases, cancer, and Alzheimer's disease [29]. Recent studies have shown that EGCG administration in fetuses affected by PAE decreases neuronal apoptosis of the rhombencephalon, ameliorates neurogenesis processes, improves fetal growth restriction (FGR), and increases the effect of the endogenous antioxidant defense systems [30]. Additionally, EGCG improves the neuronal plasticity in Down's syndrome patients, blocking the overexpression of the Dyrk1A protein, a general inhibitor of neuronal plasticity [31].

In this study, we compare the effects of PAE on the placenta and fetal brain development in two humanlike patterns of alcohol exposure, binge and Mediterranean drinking, in a C57BL/6J mouse model. We also describe the effect of EGCG administration on oxidative stress, fetal growth, placental development, and neurogenesis processes in both patterns.

2. Results

Two hundred and eighty-three mouse fetuses were included in the study (42 Mediterranean (Med) control, 47 Med ethanol (EtOH), 45 EtOH Med+EGCG, 54 Binge (Bin) control, 44 Bin EtOH, and 47 Bin+EGCG EtOH) from 36 dams. Two hundred and seventy-nine were alive by Day 19 (cesarean section) (one fetal demise in the Med control group, one in the Bin EtOH group, and two in the Bin+EGCG EtOH group). Mean litter size was 8.2 ± 1.9 for the Med control, 7.8 ± 1.3 for the Med EtOH, 7.2 ± 1.9 for the Med+EGCG EtOH, 8.2 ± 1.9 for the Bin control, 7.3 ± 1.6 for the Bin EtOH, and 7.8 ± 1.5 for the EtOH groups.

2.1. Blood Alcohol Concentrations and Epigallocatechin-3-Gallate Determination

BACs were first measured from maternal blood samples obtained by cardiac puncture. Table 1 shows BAC determinations under the various experimental conditions. As per the NIAAA definition [20], BACs were above 0.8 g/L in the Bin EtOH and Bin + EGCG EtOH groups. For the Mediterranean groups, BACs were between 0.12 g/L in the Med EtOH and 0.32 in the Med + EGCG EtOH, three to four times less concentrated than for the binge pattern.

Table 1. Blood alcohol concentrations for the various experimental conditions.

Condition	Sample Size	nmol/uL (Mean ± SD)	g/L (Mean ± SD)
Med EtOH	4	2.69 ± 0.80	0.12 ± 0.04
Med + EGCG EtOH	5	6.94 ± 2.36	0.32 ± 0.11
Bin EtOH	4	24.03 ± 1.64	1.11 ± 0.08
Bin + EGCG EtOH	4	32.22 ± 7.24	1.48 ± 0.33

EtOH Med: 10% (*v*/*v*) of ethanol solution in tap water (0.75 g/Kg) in two administrations; Med + EGCG EtOH: ethanol 0.75 g/Kg + EGCG 30 mg/Kg in two administrations; Bin EtOH: 20% (*v*/*v*) of ethanol solution (3 g/Kg/day) once a day; Bin + EGCG EtOH: ethanol 3 g/Kg/day (30 mg/Kg/day) once a day. Med: Mediterranean pattern; Bin: binge pattern; EtOH: ethanol; EGCG: epigallocatechin-3-gallate; SD: standard deviation.

EGCG concentrations in plasma were analyzed using ultra-performance liquid chromatography electrospray tandem mass spectrometry (UPLC-ESI-MS/MS) under the various experimental conditions (EGCG alone, Bin EtOH + EGCG, and Med + EGCG EtOH) 40 min after EGCG administration. Similar intergroup results were obtained (mean ± SD: 31.5 µg/mL ± 7.8 µg/mL) comparable to concentrations reported elsewhere [32].

2.2. Fetal Growth

Fetal and placental weight were analyzed at gestational Day 19 to evaluate the impact of binge and Mediterranean drinking patterns on fetal growth, as well as the effects of EGCG coadministration during pregnancy. Figure 1 summarizes fetal and placental weights of the mice under the various experimental conditions. No significant differences

in placental weights were found in any of the evaluated groups. However, fetal weights were significantly lower in the Bin EtOH group ($p < 0.0001$) compared to the Bin control or Med EtOH groups (Kruskal–Wallis analyses; $p < 0.05$). Interestingly, similar weights were observed in the Bin + EGCG EtOH and Bin control groups, with significant differences when compared to the Bin EtOH group ($p < 0.0198$), indicating complete recovery of fetal weight in presence of the antioxidant. No fetal weight differences were found for the Mediterranean groups.

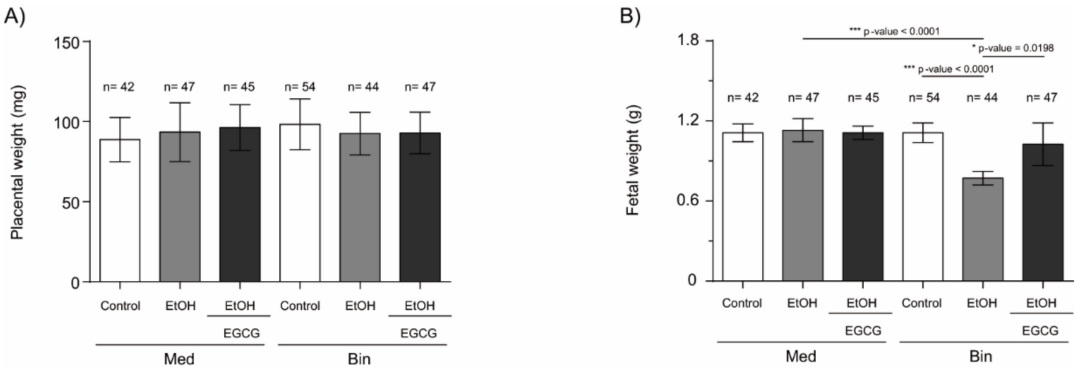

Figure 1. C57BL/6J mouse placental (**A**) and fetal (**B**) weight at gestational Day 19 under the various experimental conditions. Administered once a day: Binge (Bin) control: isocaloric maltodextrin solution (5.52 g/Kg/day); Bin EtOH: 20% (*v*/*v*) of ethanol solution in tap water (3 g/Kg/day); Bin + EGCG EtOH: ethanol 3 g/Kg/day + EGCG 30 mg/Kg/day. Administered twice a day: Med control, Med: isocaloric maltodextrin solution (1.38 g/Kg/day); Med EtOH: 10% (*v*/*v*) of ethanol solution (0.75 g/Kg); Med + EGCG EtOH: ethanol 0.75 g/Kg + EGCG 30 mg/Kg. For intergroup comparisons, the Kruskal–Wallis test was used. Asterisks denote the level of significance: * *p*-value < 0.05, *** *p*-value < 0.005

2.3. Placental Angiogenic Factors

Immunohistochemistry (IHC) and Western blot (WB) were used to determine the effect of different ethanol doses and EGCG treatment on placental tissue vasculogenesis and angiogenesis. Figure 2 shows the effect of PAE on placental VEGF-A levels. IHC assays (Figure 2A) showed a downregulation of VEGF-A, significant in the Mediterranean ($p = 0.02$) and binge ($p = 0.001$) drinking patterns (Kruskal–Wallis test, Dunn's correction for multiple comparisons). Additionally, significant increase in VEGF-A levels were observed for the Med EtOH ($p = 0.02$) and Bin EtOH ($p = 0.002$) groups treated with EGCG in comparison to the Med EtOH and the Bin EtOH group, respectively (Figure 2A). WB results were similar to the IHC in both EtOH groups in comparison to the controls; a statistically significant decrease was found for the Med EtOH group ($p = 0.002$) (Figure 2B). As in the IHC, WB measurements showed statistically significant higher expression of VEGF-A in the Med EtOH group treated with EGCG ($p = 0.03$) and a slight trend in the Bin EtOH treated group in comparison to the EtOH groups.

Figure 3 shows PLGF expression as per IHC and WB analysis of placentas. No significant differences were found in PLGF quantification neither in the immunostaining of placental tissues nor in WB analyses for the experimental groups for the Med EtOH and Bin EtOH groups (Figure 3A,B). However, WB revealed a significant increase in PLGF levels ($p = 0.004$) for the Bin + EGCG EtOH group in comparison to the Bin EtOH group (Figure 3B).

The effects of PAE on the VEGF-R1 antiangiogenic factor is shown in Figure 4. WB analysis showed an increase in placental VEGF-R1 expression for the Med EtOH and Bin EtOH groups, with no significant differences (Figure 4B). However, EGCG rescued VEGF-R1 expression in the Med EtOH ($p = 0.0001$) and Bin EtOH ($p = 0.005$) groups (Figure 4B). Surprisingly VEGF-R1 expression increased in presence of EGCG in both EtOH groups, as confirmed by the immunostaining results (Figure 4A).

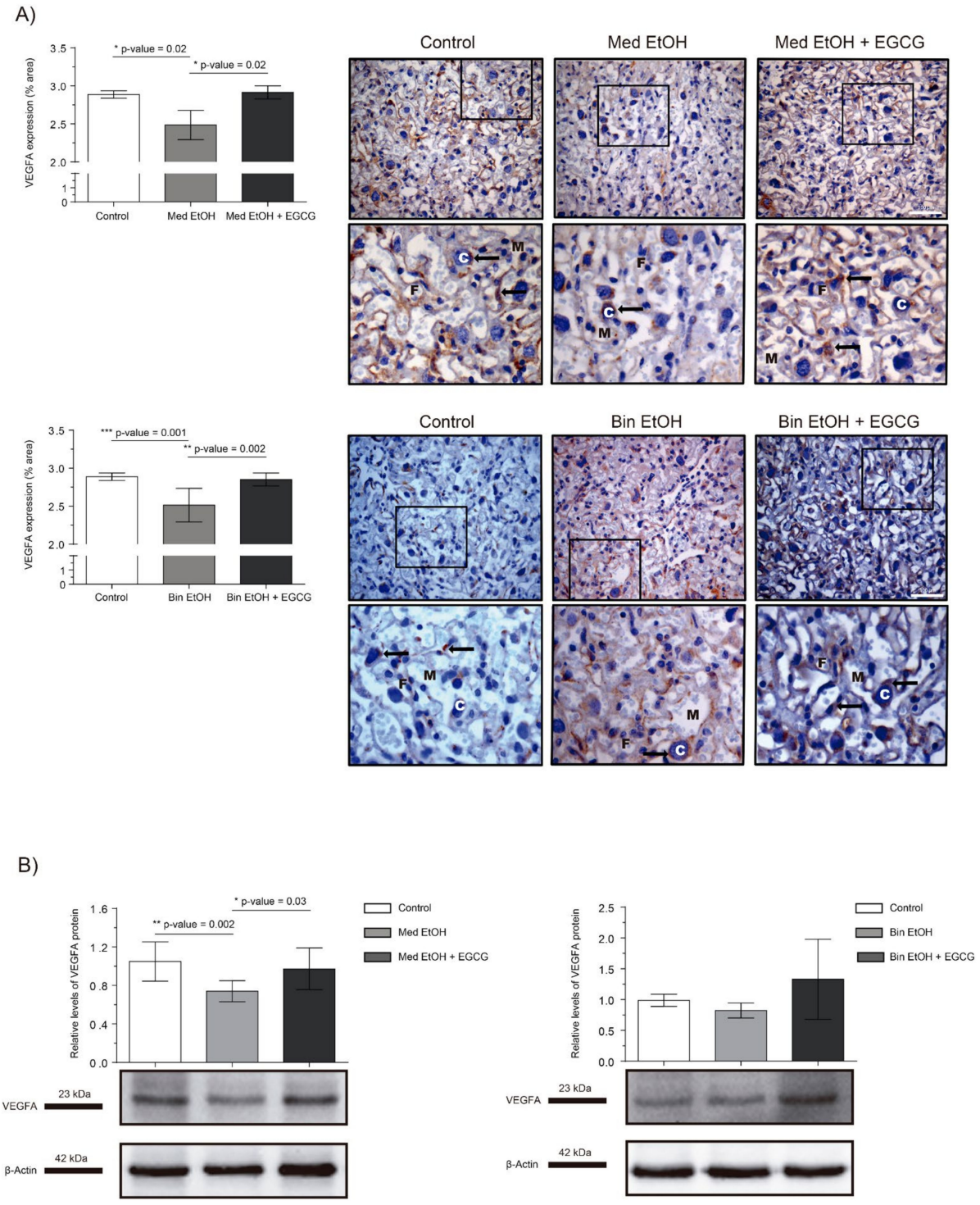

Figure 2. Representative vascular endothelial growth factor A (VEGF-A) immunostaining of placenta sections (**A**) and Western blot VEGF-A protein analysis in placental lysed samples (**B**) for prenatal alcohol exposure (PAE) and EGCG treatment for binge and Mediterranean patterns. (**A**) Boxed VEGF-A immunostaining sections represent the enlarged image of the labyrinth zone. VEGF-A quantification at two different fields (40×) per section. (**B**) Representative Western blot of VEGF-A expression observed in all analyzed samples under the various experimental conditions. Protein levels were normalized using α-tubulin as load control. Arbitrary units express "fold change". At least five samples from different litters were used from each group. For intergroup comparisons, the Kruskal–Wallis test (Dunn's test for multiple comparisons) was used. Asterisks denote the level of significance: * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.005. M: maternal blood; F: fetal blood; C: cytotrophoblast; arrows: VEGF-A positive staining.

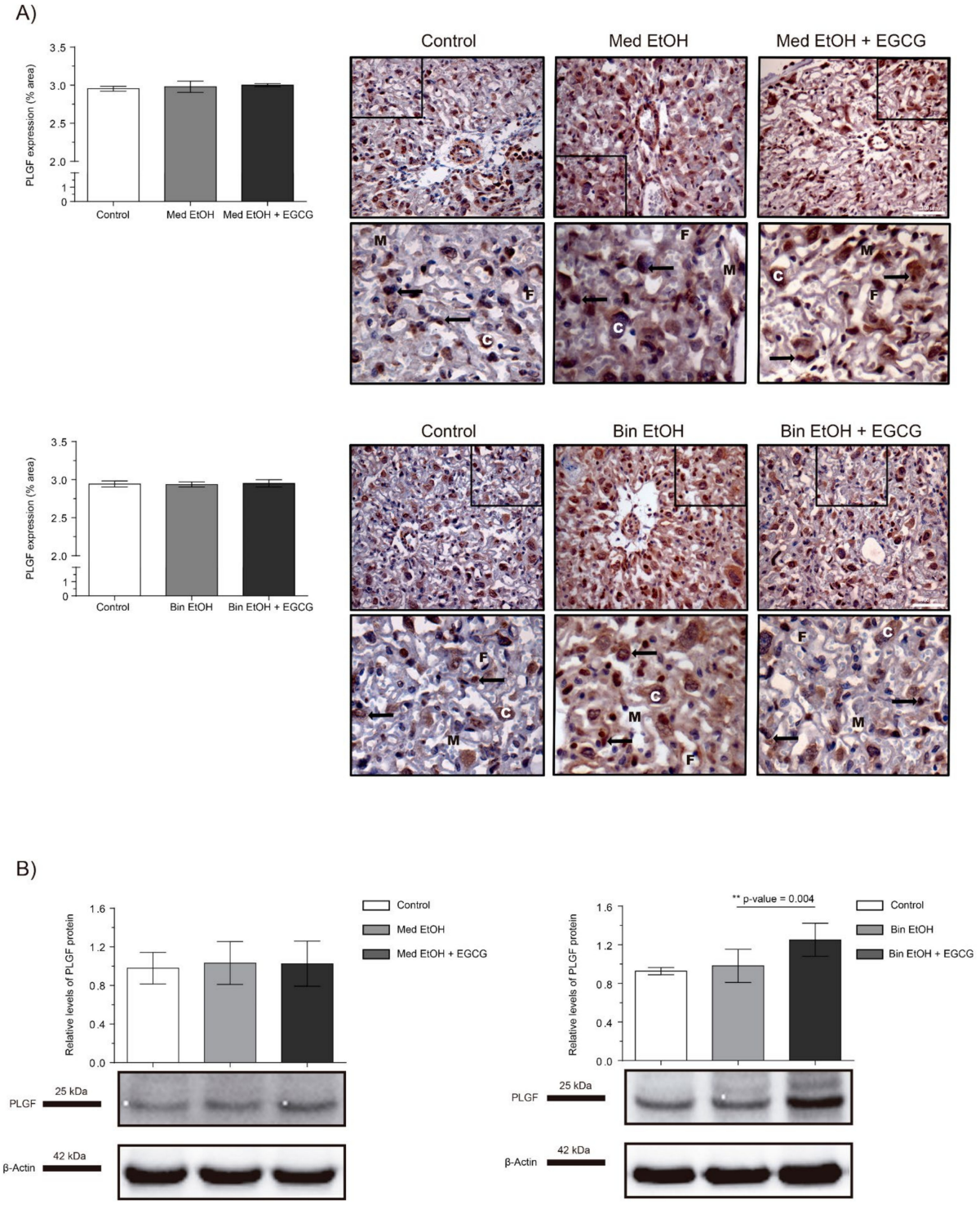

Figure 3. Representative placental growth factor (PLGF) immunostaining in placenta sections (**A**) and Western blot PLGF protein analysis in placental lysed samples (**B**) for PAE and EGCG treatment for binge and Mediterranean patterns. (**A**) Boxed sections in placental growth factor protein immunostaining represent an enlarged image of the labyrinth zone. PLGF protein quantification for two different fields (40×) per section. (**B**) Representative Western blot of PLGF protein expression observed in all samples analyzed under the various experimental conditions. Protein levels were normalized using α-tubulin as load control. Arbitrary units express "fold change". At least five samples from different litters were used from each group. For intergroup comparisons, the Kruskal–Wallis test (Dunn's test for multiple comparisons) was used. Asterisks denote the level of significance: ** *p*-value < 0.01. M: maternal blood; F: fetal blood; C: cytotrophoblast; arrows: PLGF positive staining

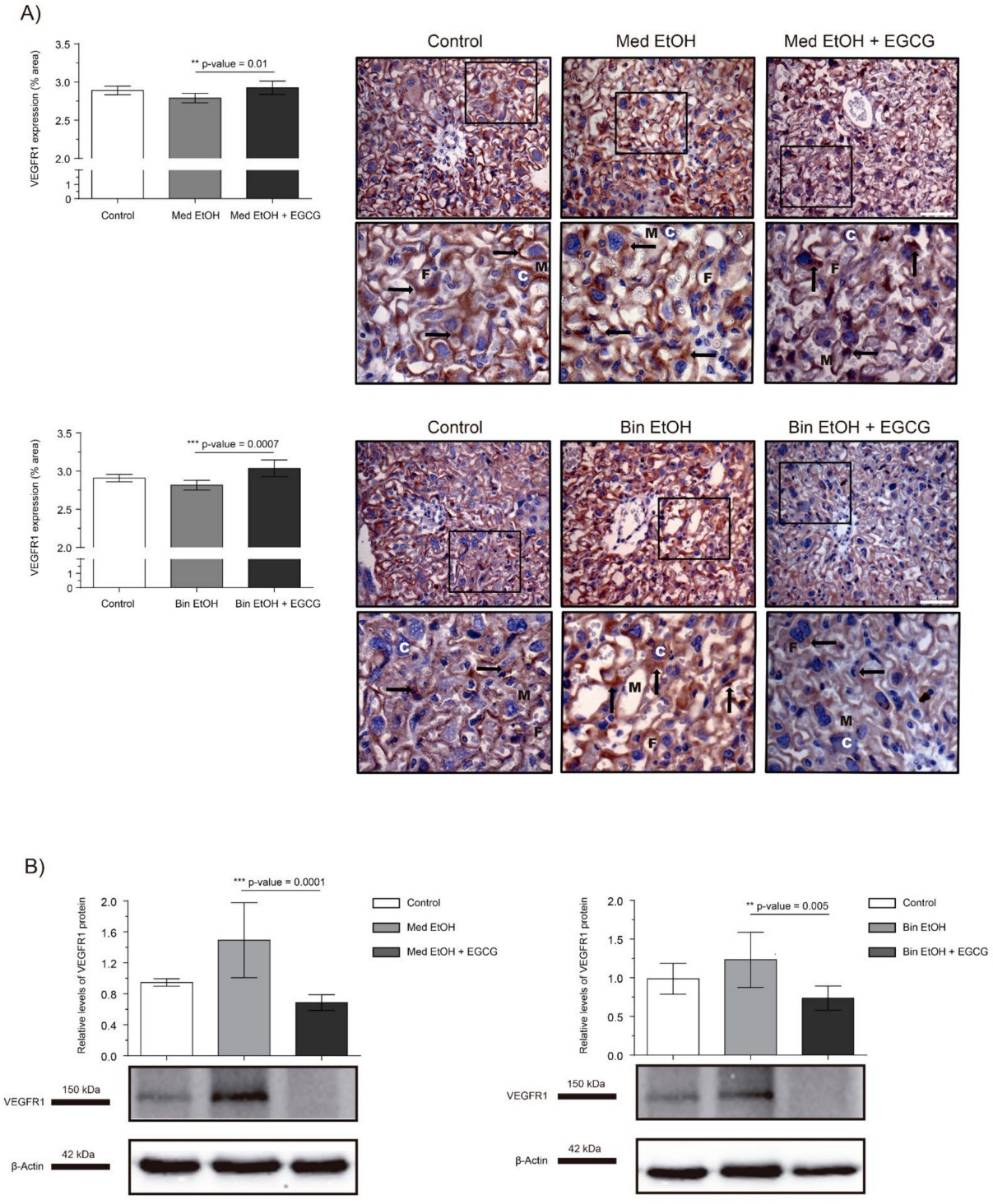

Figure 4. Representative vascular endothelial growth factor receptor 1 (VEGF-R1) immunostaining in placenta sections (**A**) and Western blot VEGF-R1 protein analysis in placental lysed samples (**B**) for PAE and EGCG treatment binge and Mediterranean experimental conditions. (**A**) Boxed sections in VEGF-R1 immunostaining are shown an enlarged image of the labyrinth zone. VEGF-R1 quantification in placental sections in two different fields (40×) per section. (**B**) Representative Western blot of VEGF-R1 expression observed in all samples analyzed for the different experimental conditions. Protein levels were normalized using α-tubulin as load control. Arbitrary units express "fold change". At least five samples from different litters were used in each group. Kruskal–Wallis test (Dunn's test for multiple comparisons) was used for inter-group comparisons. Asterisks denote the level of significance: ** *p*-value < 0.01, *** *p*-value < 0.005. M: maternal blood; F: fetal blood; C: cytotrophoblast; arrows VEGF-R1 positive staining.

2.4. Effect of Ethanol on Oxidative Stress

We assessed the effects of PAE and EGCG treatment on fetal brain oxidative stress by measuring the levels of the Nrf2 transcriptional factor, a key regulator of oxidative stress response. Immunofluorescence of the dentate gyrus (DG) of the hippocampus and cerebellum revealed no significant changes in Nrf2 expression in the Med EtOH group; similar results were obtained with WB analysis (Figure 5A–C). However, a slight increase in Nrf2 levels was found in the DG for the Bin EtOH group (Figure 5A) in comparison to the controls, with no statistical significance (Kruskal–Wallis test). Similar results were observed with the WB analysis (Figure 5C): no differences between the Mediterranean groups and a nonsignificant increase in the Bin EtOH group compared to the control. Interestingly, reduced levels of oxidative stress were observed with EGCG treatment in the DG for the Bin EtOH group ($p = 0.001$) (Figure 5A). Similar findings were found with the WB analysis ($p = 0.007$) (Figure 5C).

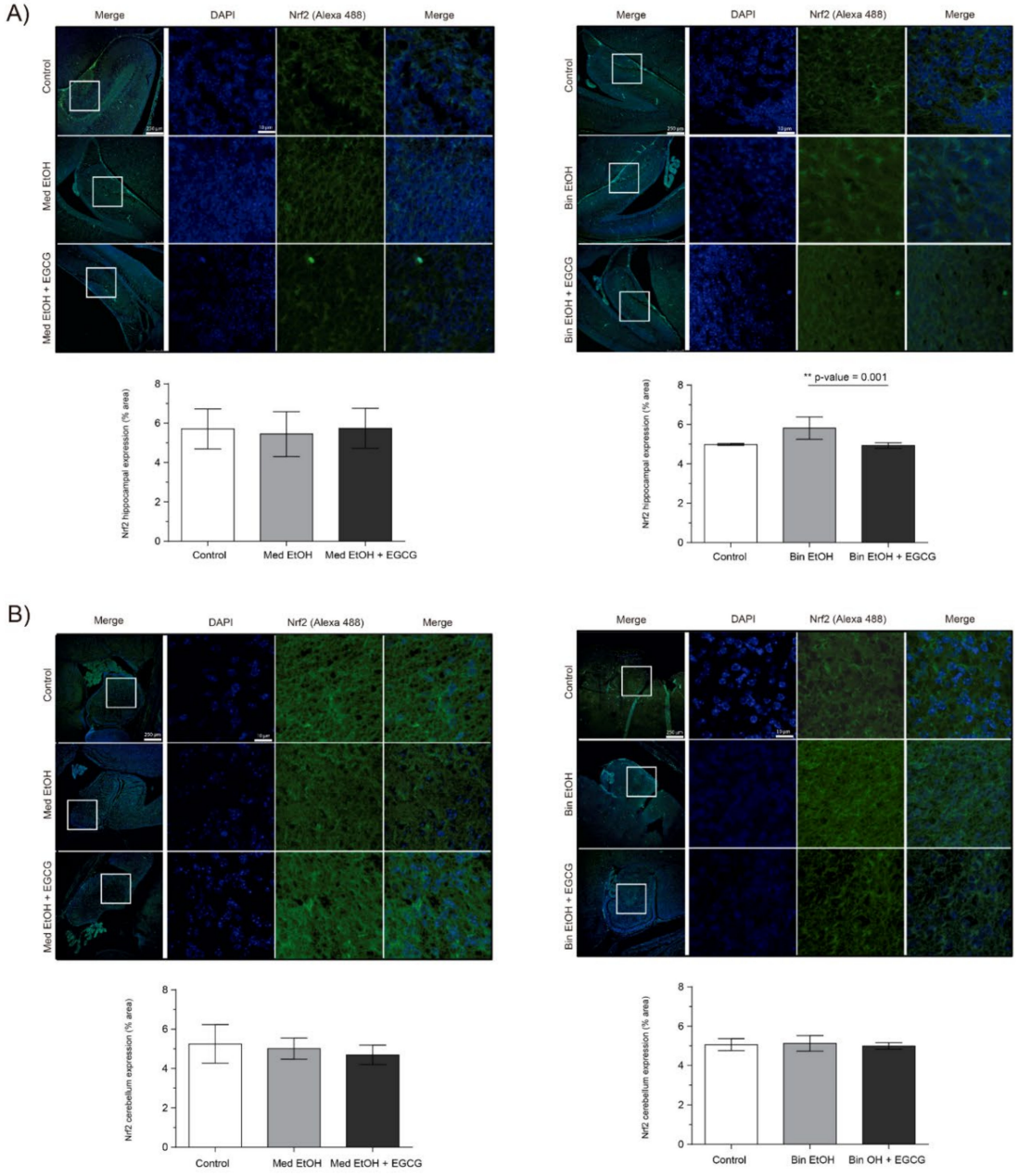

Figure 5. *Cont.*

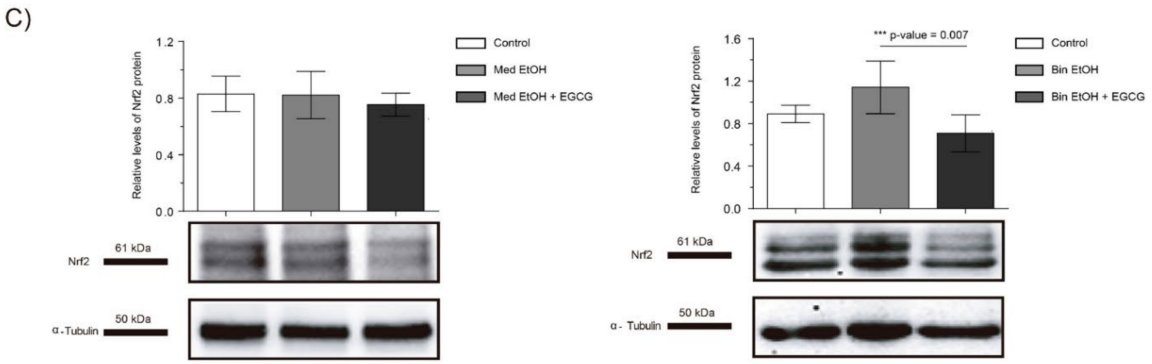

Figure 5. Nuclear factor erythroid 2-related factor 2 (Nrf2) immunofluorescence (Alexa 488 in green for the Nrf2 biomarker, and 4′,6-diamidino-2-phenylindole (DAPI) to stain the nuclei in blue) in the hippocampus (**A**) and cerebellum (**B**). Boxed regions in the hippocampus and cerebellum are shown at higher magnification using 63× Oil Immersion. Nrf2 protein analysis in brain lysed samples (**C**) under the selected experimental conditions (PAE and EGCG treatment). (**A**) Nrf2 quantification in whole sections of the dentate gyrus using a 10× objective lens. (**B**) Nrf2 quantification in whole sections of the cerebellum using a 10× objective lens. (**C**) Representative Western blot of Nrf2 expression analyzed under the various experimental conditions. Protein levels were normalized using α-tubulin as load control. Arbitrary units express "fold change". At least five samples from different litters were used from each group. For intergroup comparisons, the Kruskal–Wallis test (Dunn's test for multiple comparisons) was used. Asterisks denote the level of significance: ** p-value < 0.01. *** p-value < 0.005.

2.5. Neuronal Maturation

NeuN and DCX were chosen as representative biomarkers of neural maturation to assess the effects of PAE and EGCG therapy on these processes during fetal brain organogenesis. Similar results in neuronal nuclei quantification were obtained for IHC staining (Figure 6A for DG, Figure 6B for cerebellum) and WB analysis (Figure 6C). PAE caused a decrease of mature neurons in both types of maternal drinking patterns compared to the controls. NeuN$^+$ neurons in the DG and in the cerebellum of the Med EtOH group ($p < 0.05$) and in the cerebellum of the Bin EtOH group ($p < 0.01$) were significantly lower than in the control groups (Dunn's test). Moreover, EGCG treatment in PAE groups led to a recovery of NeuN levels. NeuN expression was significantly higher in the EGCG-treated Med EtOH and Bin EtOH groups compared to the Med EtOH and Bin EtOH groups not treated with EGCG ($p < 0.05$ in all cases).

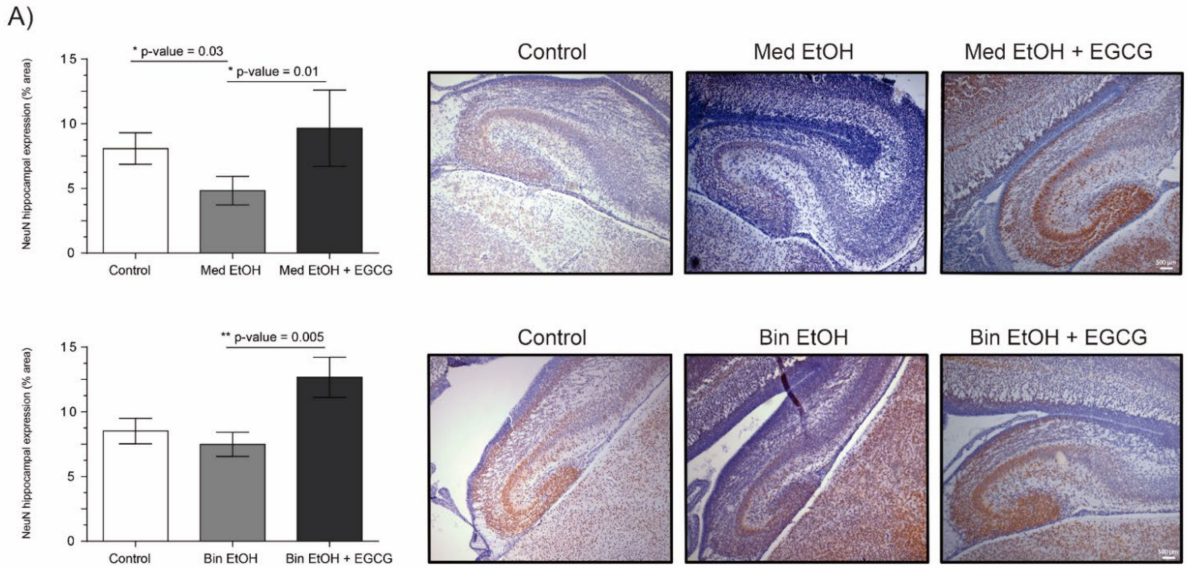

Figure 6. *Cont.*

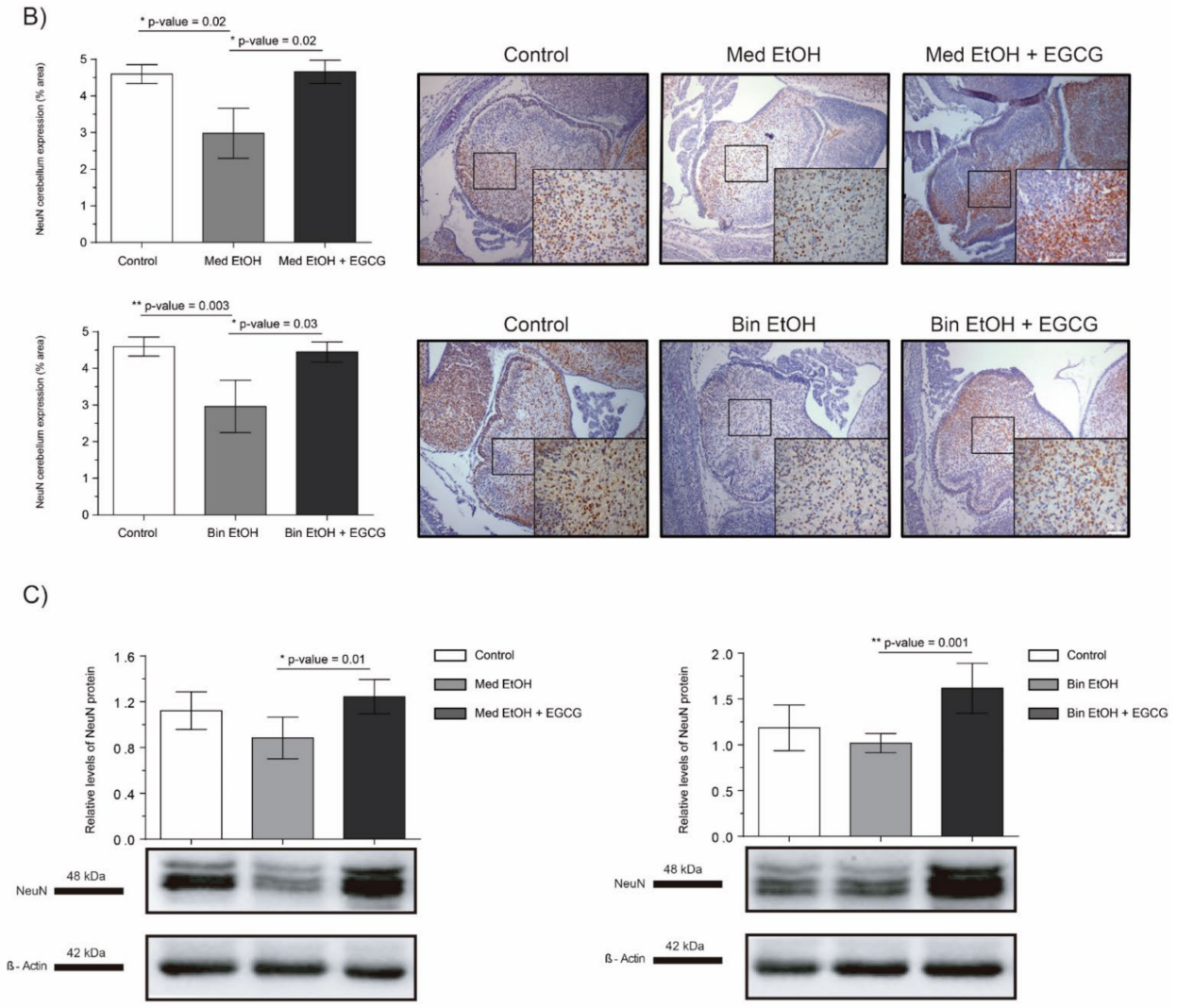

Figure 6. Representative neuronal nuclear antigen (NeuN) immunostaining in the dentate gyrus (**A**) and cerebellum (**B**), and NeuN protein analysis in whole brain lysed samples by Western blot (**C**) for PAE and EGCG treatment for the binge and Mediterranean patterns. (**A**) NeuN$^+$ cell quantification in whole sections of the dentate gyrus using a 10× objective lens, (**B**) NeuN$^+$ cell quantification in cerebellum sections in two different microscopic fields using a 40× objective lens, and (**C**) Representative Western blot of NeuN expression observed in all analyzed samples under the various experimental conditions. Protein levels were normalized using α-tubulin as load control. Arbitrary units express "fold change". At least five samples from different litters were used from each group. For intergroup comparisons, the Kruskal–Wallis test (Dunn's test for multiple comparisons) was used. Asterisks denote the level of significance: * p-value < 0.05, ** p-value < 0.01.

Representative results of doublecortin (DCX) immunoreactive (IR) cells in the DG and cerebellum are shown in Figure 7, as well as the levels of DCX in whole fetal brain extracts by WB. Immunostaining of the DG of the hippocampus (Figure 7A) showed significant increase of immature neurons in the Med EtOH and Bin EtOH groups compared to the control groups ($p < 0.005$) (Kruskal–Wallis test). DCX levels in the Bin EtOH and Med EtOH groups decreased with EGCG treatment, the latter being statistically significant ($p < 0.05$) in comparison to the controls. Quantification of doublecortin-immunoreactive (DCX-IR) neurons in the cerebellum (Figure 7B) revealed no statistical significant differences in any of the experimental groups. According to the WB immunoassay (Figure 7C), quantification of immature neurons was higher for the Bin EtOH and Med EtOH groups compared to the controls, with statistical significance for the Bin EtOH group ($p < 0.05$). Interestingly, the levels of DCX were significantly lower in the EGCG-treated Bin EtOH and Med EtOH groups ($p < 0.001$), indicating a partial recovery of the PAE phenotype. Brains prenatally

exposed to alcohol showed an increase of immature neurons, being more pronounced in specific brain areas, such as the DG. This effect may be partially counterbalanced with EGCG treatment.

2.6. Astrocyte Differentiation

The analysis of the glial fibrillary acidic protein (GFAP) allowed to assess glial cell to astrocyte differentiation in PAE fetal brains and the effects of EGCG cotreatment in brains prenatally exposed to alcohol. In spite of the trend towards a reduction of GFAP levels after PAE observed in the DG in WB and immunofluorescence, statistical significance in the results were only confirmed for the Bin EtOH group in comparison to the controls ($p = 0.046$) in WB assays (Figure 8C) (Kruskal–Wallis test). However, a clear and significant increase in the EGCG cotreatment groups (Med EtOH+EGCG and bin EtOH+EGCG) was observed for IHC (DG and cerebellum) (Figure 8A–C), as well as for whole fetal brain WB experiments. Interestingly, GFAP levels were higher in presence of EGCG in comparison to the control groups in these series of experiments (Figure 8A–C).

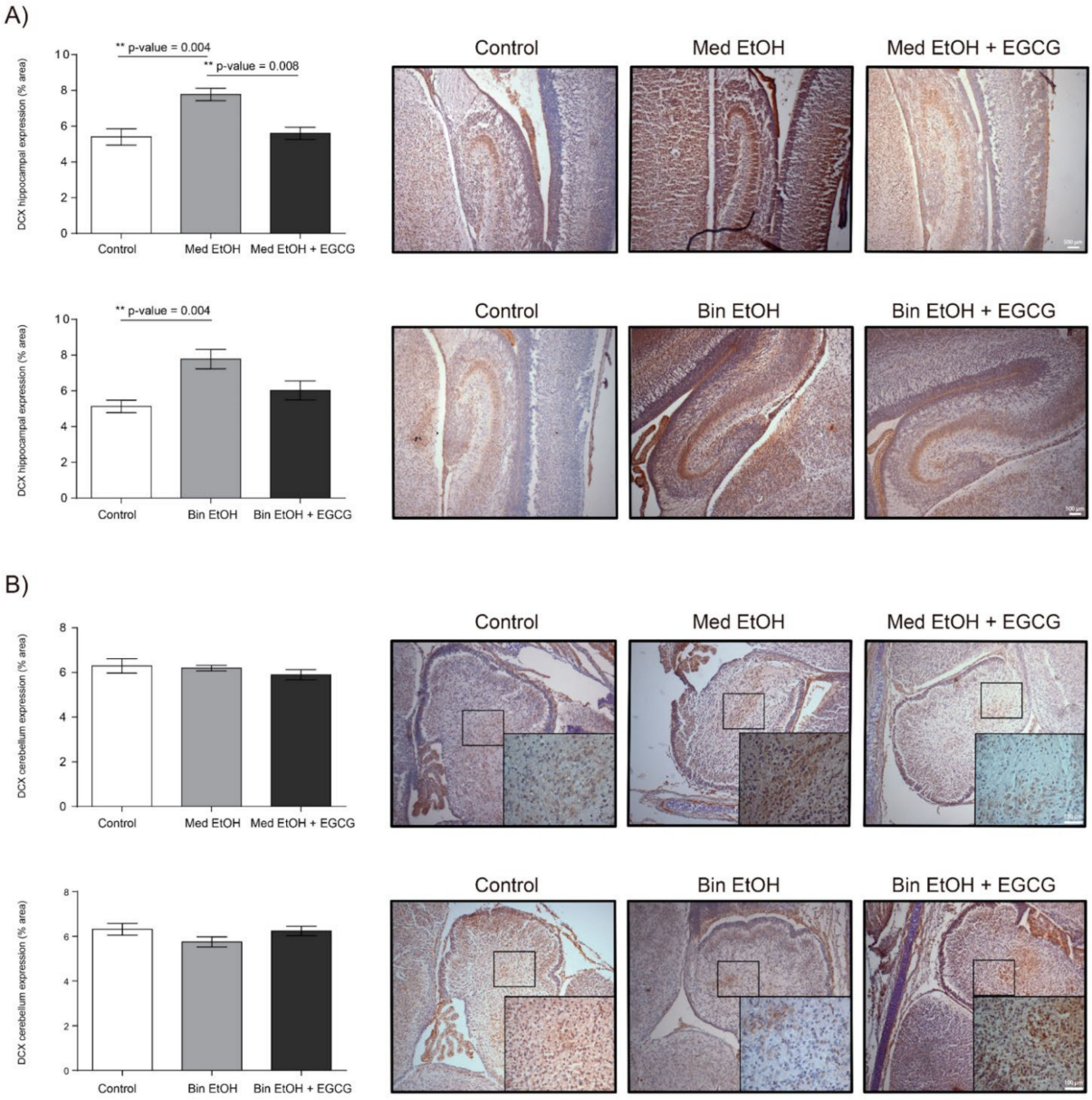

Figure 7. *Cont.*

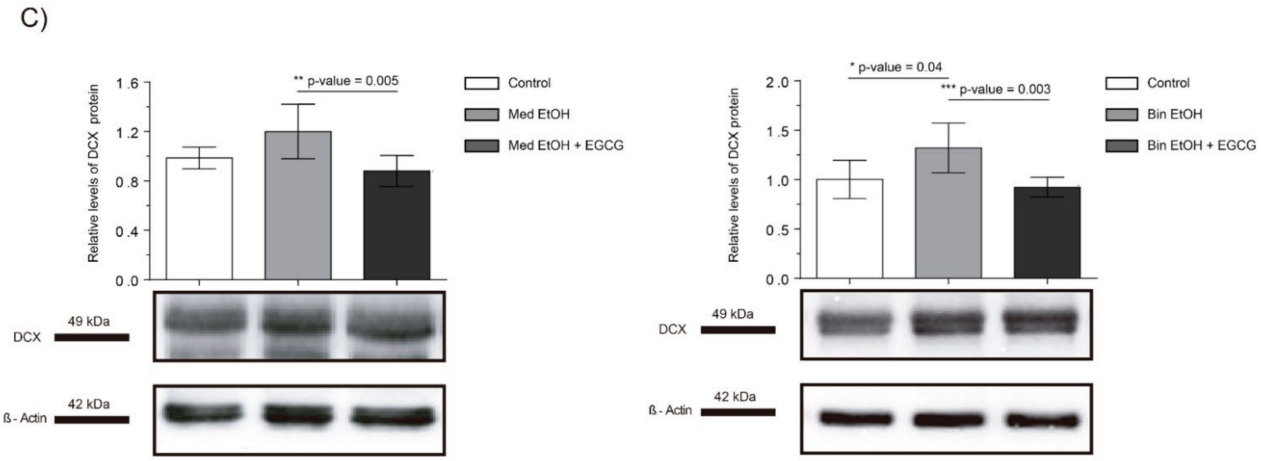

Figure 7. Representative doublecortin (DCX) immunostaining in the hippocampus (**A**) and cerebellum (**B**), and Western blot DCX protein analysis in whole brain lysed samples (**C**) for PAE and EGCG treatment. (**A**) DCX^+ cell quantification in whole sections of the dentate gyrus using a 10× objective lens. (**B**) DCX^+ cell quantification in sections of the cerebellum in two different microscopic fields using a 40× objective lens. (**C**) Representative Western blot of DCX expression observed in all analyzed samples for the various experimental conditions. Protein levels were normalized using α-tubulin as load control. Arbitrary units express "fold change". At least five samples from different litters were used from each group. Kruskal–Wallis test (Dunn's test for multiple comparisons) was performed for inter-group comparisons. Asterisks denote the level of significance: * p-value < 0.05, ** p-value < 0.01. *** p-value < 0.005.

2.7. Neuronal Plasticity

The brain-derived neurotrophic factor (BDNF) is a widely distributed neurotrophin in the central nervous system (CNS) and a classical marker of neural plasticity. We selected the BDNF as biomarker to assess trophic support and synaptic plasticity. Immunostaining of the DG of the hippocampus (Figure 9A) and cerebellum (Figure 9B) showed no statistically significant differences in BDNF levels neither in the EtOH nor the EtOH + EGCG groups in comparison to the controls. Similar results were obtained for the WB analyses (Figure 9C).

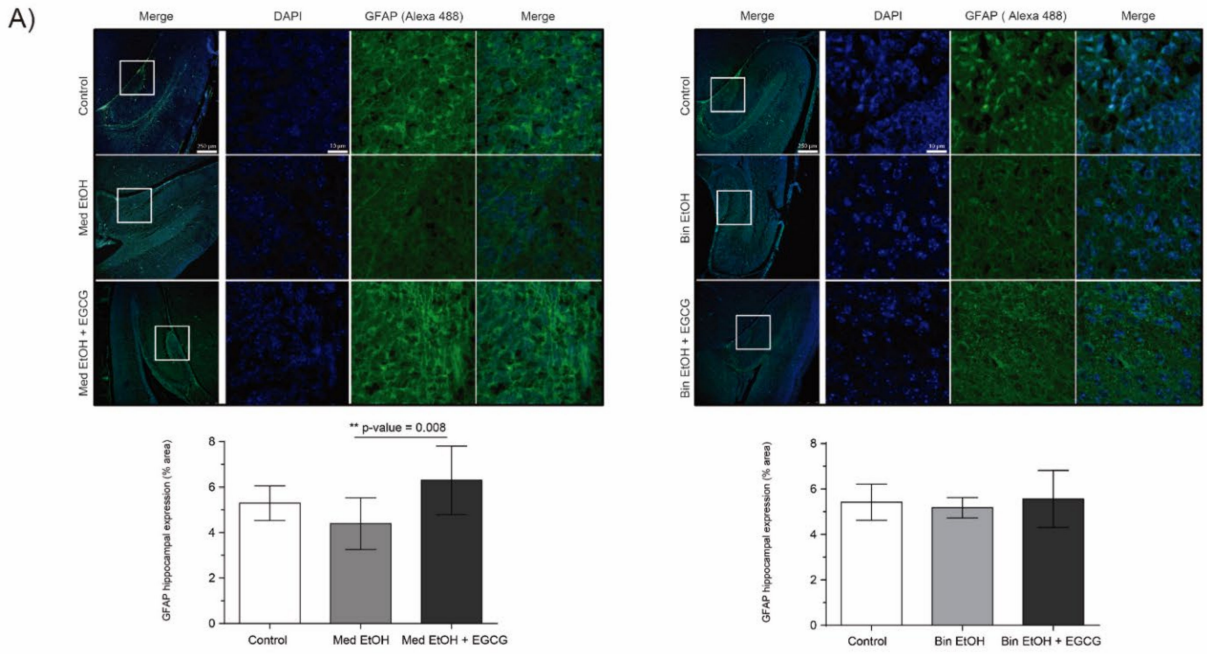

Figure 8. *Cont.*

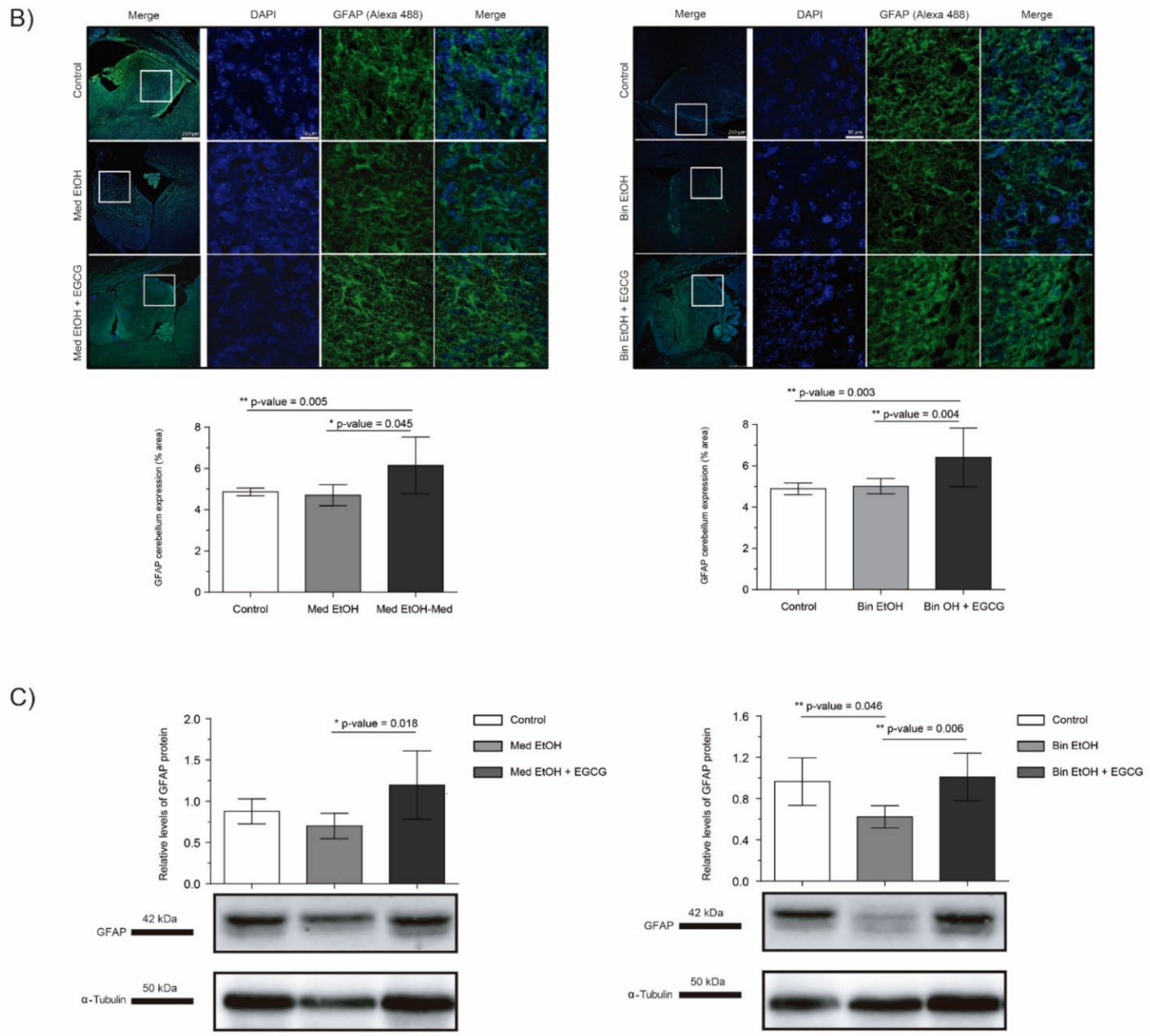

Figure 8. Glial fibrillary acidic protein (GFAP) immunofluorescence (Alexa 488 in green for the GFAP biomarker and DAPI to stain the nuclei in blue) in the hippocampus (**A**) and cerebellum (**B**). Boxed regions in hippocampus and cerebellum are shown at higher magnification using 63× Oil Immersion. Glial fibrillary acidic protein analysis in brain lysed samples (**C**) for PAE and EGCG treatment under the selected experimental conditions. (**A**) GFAP quantification in whole sections of the dentate gyrus using a 10× objective lens. (**B**) GFAP quantification in whole sections of the cerebellum using a 10× objective lens. (**C**) Representative Western blot of GFAP expression analyzed under the various experimental conditions. Protein levels were normalized using α-tubulin as load control. Arbitrary units express "fold change". At least five samples from different litters were used from each group. For intergroup comparisons, the Kruskal–Wallis test (Dunn's test for multiple comparisons) was used. Asterisks denote the level of significance: * *p*-value < 0.05, ** *p*-value < 0.01.

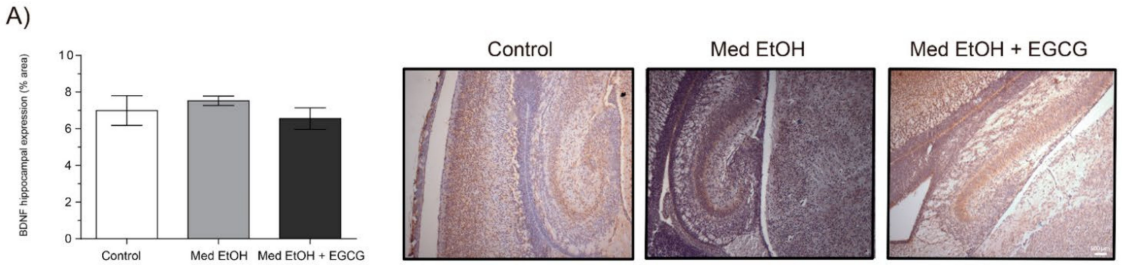

Figure 9. *Cont*.

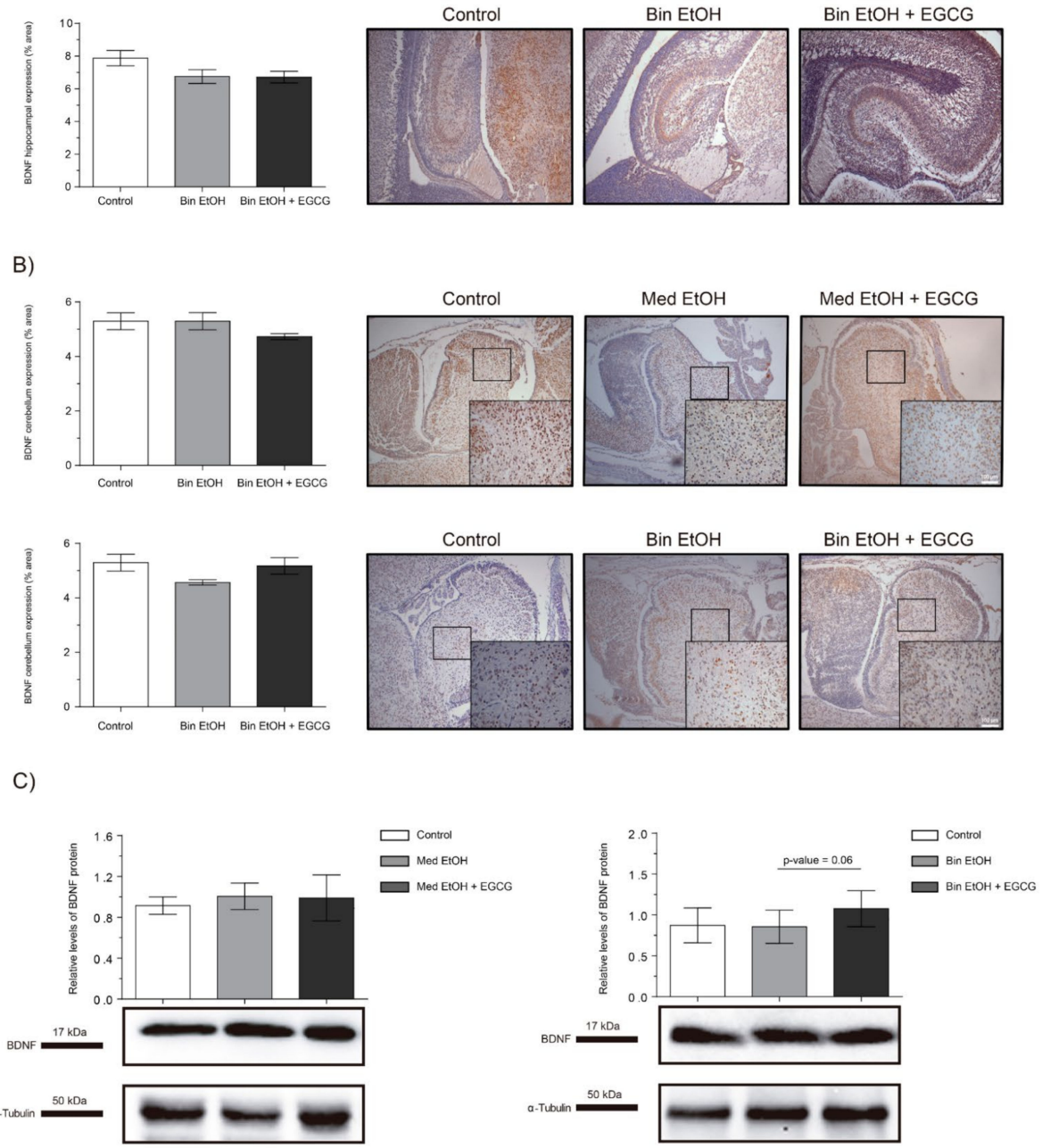

Figure 9. Representative brain-derived neurotrophic factor (BDNF) immunostaining in the hippocampus (**A**) and cerebellum (**B**), and Western blot BDNF protein analysis in whole brain lysed samples (**C**) for PAE and EGCG treatment in the binge and Mediterranean patterns. (**A**) BDNF quantification in the dentate gyrus whole sections using a 10× objective lens. (**B**) BDNF quantification in the cerebellum sections in two different microscopic fields using a 40× objective lens. (**C**) Representative Western blot of BDNF expression observed in all samples analyzed under the various experimental conditions. Protein levels were normalized using α-tubulin as load control. Arbitrary units express "fold change". At least five samples from different litters were used for each group. For intergroup comparisons, the Kruskal–Wallis test (Dunn's test for multiple comparisons) was used.

3. Discussion

Our results support EGCG as a promising antioxidant therapy to attenuate the consequences of prenatal alcohol exposure. Antioxidant therapy may ensure proper development of the placenta and fetal growth. Additionally, the prenatal effect of EGCG on neural maturation and differentiation processes may lead to normal neurodevelopment, improving behavioral and cognitive outcomes in children.

Fetal development is closely linked to placental formation. PAE exerts a negative influence on placentation and leads to FGR [4,33]. In Figure 1, fetuses exposed to high doses of alcohol show lower fetal weights compared to controls, demonstrating the inverse relation between fetal growth and PAE. Our results are in line with previous studies that report FGR on fetuses prenatally exposed to ethanol [33] and the dose-dependent effects of PAE on fetal weight [4]. The expression of angiogenic factors follows dynamic patterns according to the gestational stage to address metabolic requirements. Moreover, PAE produces an imbalance of the expression of these angiogenic factors with the consequent abnormal placental development [34,35]. Downregulation of VEGF-A (Figure 2), a regulator of angiogenesis and vascular permeability, is seen in placentas from fetuses prenatally exposed to ethanol in Mediterranean and binge human drinking patterns. These results reveal the deleterious effect of any type of alcohol consumption during pregnancy on placental angiogenesis. Similarly, decreased levels of placental VEGF with PAE have previously been reported by other authors [34]. Moreover, our study shows an increase in the expression of VEGF-R1, a negative regulator of embryonic angiogenesis, after continued PAE (Figure 4). This increase may be responsible for the abnormal angiogenesis of the placenta. Other authors reported discordances in the expression of VEGF-R1 according to the timing of PAE. Ventureira et al. report similar results to the presented in this study when PAE occurs until GD10 [34], while Lecuyer et al. show a down-regulation of placental VEGF-R1 in ethanol treated mice for the second trimester equivalent [7]. Oxidative stress may be the main cause of the findings [34,36]. Conversely, PLGF is a member of the VEGF family and a key factor in angiogenesis and vasculogenesis during embryogenesis, and is highly regulated depending on the developmental stage [35]. In our study, there are no differences in PLGF levels at GD19 in the different groups after continued PAE (Figure 3). An upregulation of placental PLGF levels in response to PAE in the early stages of pregnancy may promote placental permeability [35] showing a progressive decrease [7] comparable to controls in later stages of pregnancy (when vasculogenesis is completed) such as seen in this work. Alterations in the VEGF-VEGF-R and/or in the VEGF-R1/PLGF ratio produce important imbalances in angiogenesis [37], which are responsible for placental disorders and FGR. In addition, PAE-related oxidative stress also contributes to angiogenesis deregulation and FGR. EGCG (an antioxidant tested in pathologies related to oxidative stress [29]) is a potential therapeutic candidate for FASD. In our study, EGCG ameliorates FGR produced by PAE and attenuates ethanol-related changes in VEGF-A and VEGF-R1 expression, partially restoring the imbalance produced by PAE. Overall, EGCG exerts positive effects on the placenta and fetal growth in all ethanol exposed groups, more evident in the binge drinking group when VEGF-A, VEGF-R, PLGF, and fetal growth were analyzed. In this context, EGCG may be a therapeutic option to maintain adequate vascularization and promote a correct angiogenesis [38] necessary for proper fetal growth.

As previously mentioned, PAE boosts the production of ROS and the dysregulation of the antioxidant systems, being one of the leading causes of FASD physiopathology [17]. Under oxidative stress conditions, Nrf2 is released from its inhibitor Keap-1, translocated from the cytoplasm to the nucleus where it triggers the expression of genes encoding antioxidant proteins and detoxifying enzymes as catalase, superoxide dismutase, and the glutathione peroxidase families [39] by binding to the antioxidant responsive elements located in the promoter region of these detoxifying enzymes. GSK-3β interacts with Nrf2, inhibiting its function and promoting Nrf2 degradation in the oxidative stress delayed response [40] and acting as an Nrf2 regulator when this molecule is accumulated in the cell. In this study, we observe that the intake of high doses of ethanol during fetal development increases the levels of the Nrf2 antioxidant response, being the DG the target for oxidative stress damage. Moreover, EGCG therapy restores Nrf2 expression to levels that are comparable to those of controls. However, no differences are observed in Nrf2 expression in the Med EtOH group (Figure 5). The protective response of Nrf2 towards oxidative stress has been widely studied in adults, but studies in fetal life are limited.

A previous study shows increased Nrf2 levels in the brains of mice embryos prenatally exposed to ethanol, similar to those determined in the Bin EtOH group [39]. Moreover, EGCG upregulates Nrf2 expression after different oxidative stress insults [41,42]. However, to date no studies have been performed on the effects of EGCG on Nrf2 in PAE. Our results indicate that with PAE, Nrf2 is upregulated and the presence of EGCG not only does not increase these levels but it also reduces Nrf2 to physiological levels to avoid a pathologic accumulation of Nrf2.

Oxidative stress and the generation of ROS are key factors in FASD neurologic manifestations [43]. In addition to its antioxidant effect, EGCG has neuroprotection properties that include iron-chelation mediated by the hypoxia-inducible factor (HIF-1α) [44] or the induction of neurite outgrowth and differentiation through the protein kinase C pathway [45]. During pregnancy, the CNS has vulnerable periods sensitive to alcohol damage, which affect the developmental processes. Our data reflect the loss of mature neurons (NeuN biomarker, Figure 6), statistically significant in the DG and cerebellum in fetuses at GD19, in both the Mediterranean and binge drinking patterns. Loss of mature neurons in the DG after alcohol exposure has been analyzed in previous studies with contradictory results depending on the moment of ethanol exposure [46,47]. Based on these findings, the timing of exposure during fetal development may be a key factor for neuronal maturation. Our study demonstrates that alcohol-related reduction of mature neurons already occurs during fetal development. Further research is necessary to evaluate neurological and behavioral alcohol-related disorders secondary to the neuronal loss in the DG and cerebellum during fetal development. In the same line, DCX is expressed by neuronal precursor cells and immature neurons in the embryonic and adult brain. Our study shows an increase of DCX+ neurons, particularly in the DG, in the Mediterranean and the binge drinking patterns (Figure 7). Elibol-Can et al. showed similar results in the different hippocampal regions after PAE according to the binge drinking pattern during the second trimester equivalent [46]. In contrast, lower DCX quantification in the DG has been reported in comparison to the controls in adult mice prenatally exposed to ethanol [48]. Increased signaling in immature neurons during fetal life may indicate a delay in the maturation processes produced by the oxidative damage generated by PAE on the organogenesis processes [49,50]. As for the effect of EGCG therapy in fetal neurogenesis, our results show an improvement in maturation and differentiation processes. The expression of NeuN and DCX in the treated groups are comparable to that in the controls (Figures 6 and 7), which indicates that EGCG may exert a beneficial effect on fetal neurogenesis. Studies with natural antioxidants in fetal life are scarce. Similar effects were shown on hippocampal neurogenesis from a neuroinflammation model in adult mice, where EGCG treatment appears to be beneficial [51]. Consistently, a study in ethanol exposed adult mice demonstrated the compensatory effect of EGCG therapy on the affected immature neurons [51]. EGCG also seems to promote proliferation and differentiation, as evidenced by increased Ki67 and neuron specific enolase expression [52,53]. These findings support EGCG as a potential therapeutic compound to prevent the delay in the neurogenesis processes produced by ethanol in fetal life.

Regarding GFAP (a glial cell to astrocyte differentiation biomarker), astrocyte differentiation is reduced following PAE, reaching statistical significance in fetuses exposed to a binge drinking pattern (Figure 8), as reported in previous studies [54]. EGCG therapy elicited a recovery of GFAP to levels comparable to that of controls (Figure 8). Although there are no previous studies on the effect of EGCG on astrocyte differentiation in FASD-like animal models, results from other studies on neurodegenerative diseases show the neuroprotective effect of EGCG on astrocyte differentiation processes [55]. In vitro models also show EGCG neuronal differentiation involvement through the protein kinase C pathway [45], the inhibition of the glycogen synthase kinase-3 (GSK-3) pathway [56], or the modulation of S100B [57].

BDNF is involved in cell survival, development, and function of the CNS, and represents one of the main biomarkers of neuronal plasticity during early development [58].

EGCG has been selected as a potential pharmacological tool against FASD due to its ability to interact and inhibit neuronal plasticity inhibitors such as Dyrk1A [31] and potentiate NGF-induced neurite outgrowth [59,60]. Our study shows no statistically significant differences in BDNF levels after neither PAE nor EGCG administration. In contrast, Feng et al. report a decrease in BDNF levels in rats prenatally exposed to a binge drinking pattern from GD5-GD20, but no differences when alcohol doses are lower [61]. Similarly, Haun et al. show a down-regulation in BDNF expression after the intake of large doses of alcohol in an adult mouse model of four-day ethanol exposure and a significant ethanol consumption decrease in the established model of alcohol dependence after BDNF administration [62]. Our experimental design ensures continued alcohol exposure, which would lead to habituation to ethanol and therefore, no changes in BDNF expression. Further research would be useful to test the effects of EGCG therapy on BDNF expression in FASD-like models with not-continuous ethanol administration.

This article proposes a prenatal intervention in mice exposed to alcohol during development to attenuate the FASD phenotype. One limitation of our study is the experimental design based on continued ethanol exposure during the 19 days of pregnancy. This may lead to an alcohol exposure adaptation in some critical processes related to FASD pathophysiology; however, we consider this continuous 19-day ethanol exposure similar to the alcohol abuse in humans. Additionally, the effects of PAE that contribute to FASD in the third trimester were not considered in this study due to the above-mentioned experimental design.

To the best of our knowledge, this is the first in vivo study that shows the differences in the FASD-like phenotype based on binge and Mediterranean human drinking patterns. Interestingly, any drinking pattern (Mediterranean or binge) produces alcohol related-effects in the offspring. The binge-drinking pattern leads to effects that are more substantial on the different processes, i.e., oxidative stress, neuronal maturation and differentiation, as well as in fetal growth. Nevertheless, the effects of the Mediterranean drinking pattern on angiogenic factors are comparable to binge drinking. Fetal growth is probably preserved in the Med EtOH group because lower doses of ethanol do not reach the threshold to produce a FASD phenotype. Finally, in the present study we evaluate the consequences of PAE based on different patterns of alcohol consumption, as well as the effect of EGCG treatment examining a wide range of FASD manifestations (placental development, fetal growth, angiogenesis, oxidative stress, and neurodevelopmental processes), and show the potential role of EGCG as a pharmacological tool during fetal development.

4. Materials and Methods

4.1. Animals, Housing, and Ethical Statement

Eight-week-old male (n = 20) and female (n = 45) C57BL/6J mice were purchased from Charles River (Barcelona, Spain) and housed in the facilities of Sant Joan de Déu Hospital, University of Barcelona. Animals were housed in standard conditions at 21 ± 1 °C, 55 ± 10% relative humidity under a controlled 12 h light/dark cycle and had free access to water and standard chow. Animal procedures were approved by the Animal Experimental Ethics Committee of the University of Barcelona (23 January 2016) and were registered on the Generalitat de Catalunya, Serveis Territorials d'Agricultura, Ramaderia, Pesca i Alimentació a Barcelona (identification code: 713/15-8744) and carried out in accordance with the recommendations in the ARRIVE guidelines for the care and use of experimental animals and the U.K. Animals (Scientific Procedures) Act, 1986 and EU Directive 2010/63/EU for animal experiments.

4.2. Prenatal Alcohol Exposure and Epigallocatechin-3-Gallate Treatment

Pure absolute ethanol (ethyl alcohol, EtOH, 1000 mL) was provided from PanReac AppliChem ITW Reagents (Dublin, Ireland), maltodextrin (100% Maltodextrin Powder, Pure series®) was purchased from Bulk Powders (Essex, UK) and EGCG (Teavigo®, 94% EGCG, 150 mg, 60 Count) was provided by Healthy Origins (Pittsburgh, PA, USA).

Mice were left to acclimatize, and then matched 2:1 (female:male). Pregnant mice (determined by observing sperm plugs) were individually housed in standard plastic cages to avoid any additional stress, and randomly allocated to one of the six experimental groups: (1) Mediterranean (Med) control: isocaloric maltodextrin solution (1.38 g/Kg/day) given in two administrations (eight-hour dosing interval); (2) EtOH Med pattern: 10% (*v*/*v*) of an ethanol solution (1.5 g/Kg/day) in two administrations (eight-hour dosing interval); (3) EtOH Med and EGCG: ethanol (1.5 g/Kg/day) in two administrations (eight-hour dosing interval) and EGCG (30 mg/Kg/day) once a day with the first dose of ethanol; (4) Binge (Bin) control: isocaloric maltodextrin solution (5.52 g/Kg/day) once a day; (5) EtOH Bin pattern: 20% (*v*/*v*) of ethanol solution in tap water (3 g/Kg/day) once a day; (6) EtOH Bin and EGCG (ethanol 3 g/Kg/day and EGCG 30 mg/Kg/day) once a day. All administrations were performed via oral gavage. An isocaloric maltodextrin solution equivalent to the calorie intake of alcohol was administered to each control group. All animals had free access to water. At Day 19 of pregnancy, mice were terminally anesthetized with pentobarbital, maternal blood samples obtained using cardiac puncture, and fetuses delivered by cesarean section. Fetal and placental weights were obtained before collecting placental and brain tissue. All tissue samples were frozen in liquid nitrogen prior their storage at −80 °C. The design of the study is summarized in Figure 10.

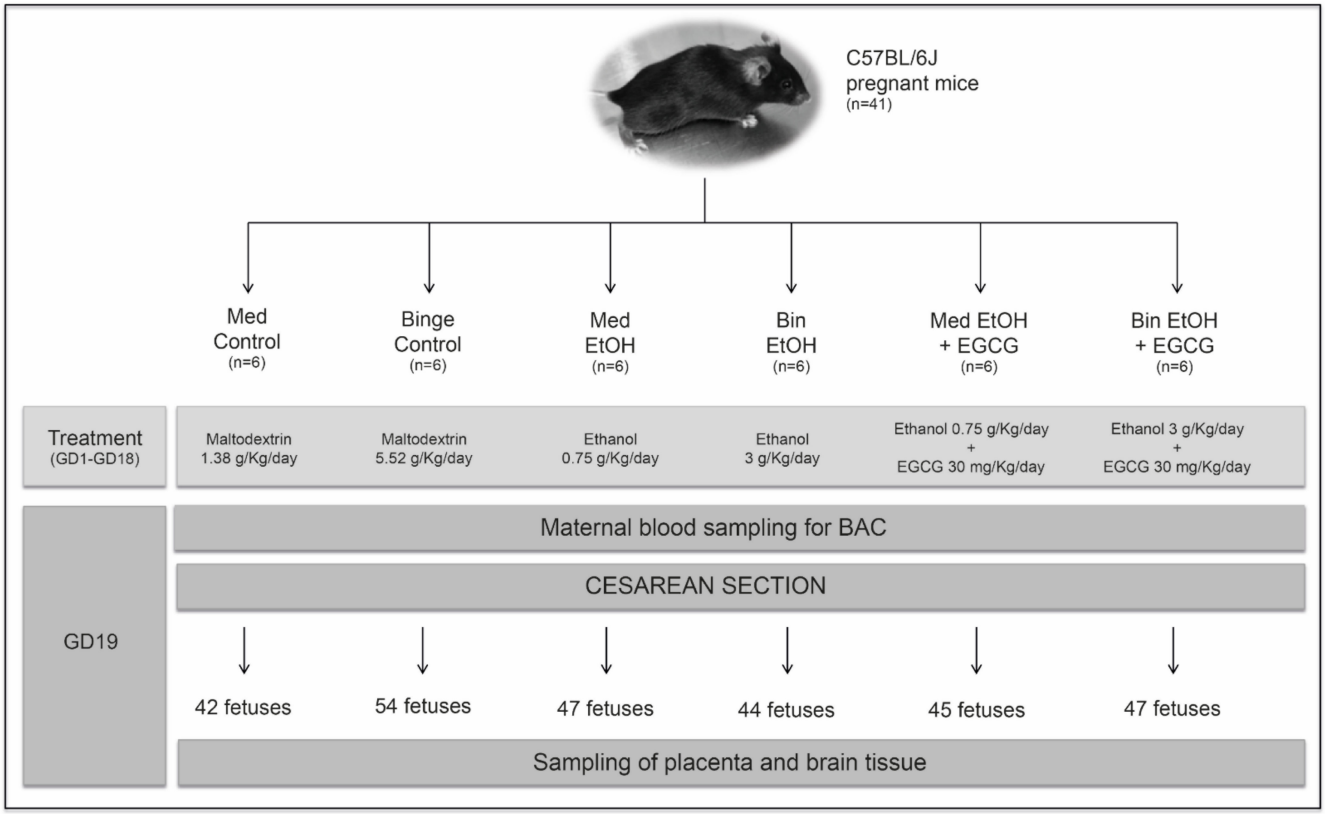

Figure 10. Flowchart of the experimental design. Sample size and doses of maltodextrin, alcohol, and epigallocatechin-3-gallate are specified for each experimental group. Maternal blood sampling to determine blood alcohol concentration and birth by cesarean section were obtained on gestational Day 19. The gestational period of the mice corresponds to the first and second human equivalent. EtOH: ethanol; Bin: binge; Med: Mediterranean; EGCG: epigallocatechin-3-gallate; GD: gestational day; BAC: blood alcohol concentration.

4.3. Reagents and Antibodies

VEGF-A (ref. SC-7269, 21 kDa monomer) was purchased from Quimigen SL (Madrid, Spain); PLGF (ref. ab180734, 25 kDa), VEGFR (ref. ab32152, 150 kDa (Flt1), NeuN (ref. ab177487, 48.5 kDa), doublecortin (ref. ab135349, 49 kDa), GFAP (ref. ab7260, 55 kDa),

and BDNF (ref. AB226843, 17 kDa) from Abcam (Cambridge, MA, USA); Nrf2 (ref. sc-722, 61 and 68 kDa) from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); Alfa-tubulin (ref. T8203, dil 1:2000, 50 kDa), beta-actin (ref. A3854, 42 kDa, dil 1:2500), and Anti Rabbit IgG secondary (ref. A0545, dil 1:2000) from Sigma-Aldrich (Sant Louis, Missouri, USA), and goat antimouse IgG (ref. G21040, dil 1:10,000) from Thermo Fisher Technologies (Waltham, MA, USA).

4.4. Blood Alcohol Concentration and Epigallocatechin-3-Gallate Determination in Pregnant Mice

One milliliter of maternal blood was collected in heparin BD Vacutainers® by intracardiac puncture 45 min post-treatment. Samples were incubated at room temperature for five minutes and then centrifuged at 1750× *g* at 4 °C for 20 min. Serum was isolated and BAC measured using the Ethanol Assay Kit (MAK076) purchased from Sigma-Aldrich. A master reaction mix (50 µL per well) containing two microliters of serum sample was performed following the recommendations of the supplier. Wells were mixed using a horizontal shaker and the reactions were incubated for 30 min at 37 °C or 60 min in the dark. Finally, measurements of absorbance at 570 nm were carried out.

The remaining serum was stored at −80 °C to evaluate EGCG concentration. For analytical purposes aliquots of serum containing EGCG (0.350 mL) were stored in Eppendorf LoBind tubes (Sarstedt 72,706,600) at −80 °C containing 20 µL of vc-EDTA (1.38 g NaH_2PO_4: H_2O, 5 g ascorbic acid, and 25 mg EDTA in 25 mL H_2O miliQ, pH 3.8) until further analysis. Samples were analyzed by the department of pharmacology of Institut Hospital del Mar d'Investigacions Mèdiques (Barcelona, Spain) following a previously published methodology [63].

4.5. Western Blot Analysis

Protein extractions were performed by mechanical tissue rupture using the Politron device (Omni Tissue Homogenizer, Omni International, Kennesaw, GA, USA) after introducing the samples in RIPA buffer (Life Technologies S.A, 89900, Carlsbad, CA, USA), using three cycles of 30 s per sample. Samples were then quantified with the DC Protein Assay kit (Bio-rad Laboratories S.A., Madrid, Spain) and absorbance measured at 780 nm (the Lowry test). After quantification, 40 µg of total protein were loaded in a volume of 30 µL per well in RIPA buffer by adding 6 µL of Loading Buffer 5× (3.125 mL 1 M Tris-HCl (pH = 6.8), 5.75 mL glycerol 87%, 1 g SDS, 1 mL β-mercaptoethanol, and 1 mL 5% bromophenol blue), heating the samples on a thermoblock (Thermo Scientific, Waltham, MA, USA) at 95 °C for protein denaturation. Electrophoresis was then performed on a 12% acrylamide gel using the molecular weight marker (precision plus protein dual color standard from BioRad, 1610374). An electric current was next applied to the electrophoresis cuvette with running buffer (3.03 g/L of Tris Base, 1.44 g/L of glycine, 1 g/L of SDS). Next, proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories SA, 162-0177) that had been previously activated five minutes in methanol, five minutes in distilled H_2O, and five minutes in transfer buffer (3.03 g/L of Tris-Base, 14.4 g/L of glycine and 200 mL/L of methanol). Protein transfer was performed in transfer buffer at 4 °C in a cold chamber for two to three hours depending on the molecular weight of the proteins. Subsequently, three five-minute washes with tris-buffered saline (TBS-T) were performed (2.4 g/L Tris-HCl pH = 7.6 and 8.8 g/L NaCl and 1 mL Tween 20); membranes were blocked with BSA 5% diluted in TBS-T for 30 min. Finally, the membranes were incubated with the primary antibody overnight at 4 °C with stirring; 1:1000 dilutions in BSA 2% were used for primary antibody incubation. The next day, the primary antibody was collected and membranes washed three times (for five minutes) with TBS-T; the secondary antimouse or antirabbit antibody was then added for two hours at room temperature with stirring. Membranes were developed with a 1: 1 mixture of the Pierce ECL WB Substrate (Cultek S.L., Madrid, Spain) in the dark using the iBright CL1000 (Invitrogen, Thermo Fisher Scientific, Cornellà de Llobregat, Spain) device. Densitometric analysis was performed to determine the intensity of the bands using the Image J program. The intensity values

obtained from the quantification were normalized with respect to the values obtained from the bands of the control protein (tubulin or actin), expressed at equal levels in all situations.

4.6. Immunohistochemistry

Fetal brain and placenta samples were fixed in 10% buffered formalin and next embedded in paraffin. Sagittal 5-µm-thick brain and transversal placenta sections were prepared, mounted on glass slides, and allowed to dry. They were next deparaffinized, unmasked, and peroxidase blocked before applying the primary antibodies. The following primary antibodies were used at the indicated dilutions: anti-VEGF-A (1:1000), anti-PLGF (1:100), anti-VEGF-R (1:200), anti-NeuN (1:700), anti-DCX (1:1500), and anti-BDNF (1:250). Slides were incubated overnight at 4 °C and then washed and incubated with the secondary horseradish peroxidase (HRP) conjugated antibodies: anti-IgG-rabbit (1:200) and anti-IgG-mouse (1:200) for one hour at room temperature. Finally, slides were visualized with diaminobenzidine and lightly counterstained with hematoxylin before being dehydrated, cleared, and mounted. The same steps were followed without the primary antibodies for negative controls; no staining was observed. Staining specificity was established by staining run simultaneously for each slide under the six experimental conditions. VEGF-A, PLGF and VEGF-R counts were carried out by selecting two center areas in the labyrinth; NeuN, and DCX counts were carried out by selecting two center areas in the cerebellar vermis and a whole area in the DG of the hippocampus from 6–10 different samples for each group. Images were captured with an Olympus light microscope using 10× and 40× magnifications. Images were obtained with a specific image software (Image ProPlus) and quantification carried out using an Image J Analysis Software and a color deconvolution algorithm to determine the percentage of positive immunostaining. Immunohistochemical staining was analyzed establishing a positive area/total area ratio (%).

4.7. Immunofluorescence

Five-micrometer-thick brain sections were prepared, mounted on glass slides, and allowed to dry. Slides were next deparaffinized, unmasked, and peroxidase blocked before applying the primary antibodies to Nrf2 (1:250) or GFAP (1:250) for one hour at room temperature. The following steps were performed in the dark: slides were incubated with the secondary antibody, a goat antirabbit IgG, coupled to Alexa Fluor-488 (1:1000) for one hour at room temperature; next, they were mounted and the nucleus stained using VectashieldR Antifade Mounting Medium with DAPI. Immunofluorescence was performed simultaneously for all histological sections of each antibody and pictures acquired the same day. Image acquisition conditions were set according to the brightest sample (exposure time, contrast and color balance) for all pictures. Nrf2 and GFAP counts were carried out by selecting two center areas in the cerebellar vermis and a whole area in the DG of the hippocampus from 6–10 different samples in each group. Images were captured using the same imaging settings for all experimental conditions and acquired with the confocal microscope Leica TCS SP5 (Leica Microsistemas S.L.U., objective 10 and 63x). GFAP and Nrf2 signals were quantified using the Image J Analysis Software. The following quantification settings for all immunofluorescence images were used: background correction was performed selecting a region of interest (ROI) in the background applying the command Proces/Math/Subtract, which subtracted the mean of the ROI plus an additional value equal to the standard deviation of the ROI multiplied by 3; the intensity of fluorescence quantification was then measured using the command Analyze/Measure and limiting to a threshold of 100 to 255 for Nrf2 and GFAP quantifications. For immunofluorescence evaluation, cerebellum and DG were analyzed establishing a positive fluorescence signal area/total area ratio (% area).

4.8. Statistical Analyses

Database management and statistical analyses of the variables were performed using the SPSS v.22 (IBM, Armonk, NY, USA) and the GraphPad (Prism, San Diego, CA, USA)

software v.6.0. For descriptive statistics, means and standard deviations (SD) were used. Intergroup comparisons were performed using the non-parametrical Kruskal–Wallis test (Dunn's correction for multiple comparisons) to analyze the differences in placental and brain protein expression. Statistical significance was set at $p < 0.05$ for all the analyses. At least three different experiments were performed to obtain the mean for each sample and at least five different samples from different litters were used in the statistical analyses.

5. Conclusions

The harmful effects of ethanol on fetal development are well known. However, few studies compare the consequences of ethanol intake considering the different patterns of human alcohol consumption. In this study, we analyze the effects of PAE on fetal growth, placenta, and neurodevelopment and show that no amount of alcohol may be considered safe during pregnancy. The timing of ethanol exposure is a key factor in FASD pathophysiology. PAE promotes alterations in placental angiogenesis, fetal growth restriction, and disorders in neuronal maturation and astrocyte differentiation processes. Furthermore, the binge drinking pattern generates the most harmful effects on the fetus. Finally, EGCG as a potential antioxidant, appears to be a safe nutraceutical option to ameliorate FASD manifestations in exposed individuals. EGCG reduces the oxidative stress generated by alcohol exposure mitigating its teratogenic effects. More studies are needed to evaluate the possible beneficial effects of the antioxidant therapy on fetal angiogenesis and neurogenesis, and the molecular pathways related to these outcomes. Finally, the results of this study need to be tested in humans to translate these findings to women that drink alcohol during pregnancy.

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Institutional Review Board Statement: The study was conducted according to the ARRIVE guidelines for the care and use of experimental animals and the U.K. Animals (Scientific Procedures) Act, 1986 and EU Directive 2010/63/EU for animal experiments, and approved by the Animal Experimental Ethics Committee of the University of Barcelona (identification code: 713/15-8744; 23 January 2016).

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Article 2: Bioavailability of epigallocatechin gallate administered with different nutritional strategies in healthy volunteers

In this article, we assessed EGCG bioavailability in humans under different nutritional conditions. The aims in this work were:

1. To analyze the bioavailability of oral EGCG administration alone or with different food supplements in healthy volunteers

2. To evaluate the pharmacokinetic parameters of EGCG in healthy volunteers

antioxidants

MDPI

Article

Bioavailability of Epigallocatechin Gallate Administered with Different Nutritional Strategies in Healthy Volunteers

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Abstract: The flavanol epigallocatechin gallate (EGCG) is being tested for the treatment of several diseases in humans. However, its bioavailability and pharmacokinetic profile needs a better understanding to enable its use in clinical trials. There is no consensus on the most appropriate concentration of EGCG in the body to obtain the maximum therapeutic effects. Therefore, the aim of this study is to analyze the bioavailability of EGCG orally administered alone or with different food supplements after overnight fasting in order to determine its optimal conditions (high concentrations in blood and the lowest interindividual variations) to be used as a pharmacological tool in human trials. Ten healthy volunteers (5 men and 5 women) aged 25 to 35 years were recruited prospectively. Three series of clinical experiments with a washout period of seven days among each were performed: (1) Teavigo® (EGCG extract) alone, (2) Teavigo® with a standard breakfast, and (3) FontUp® (Teavigo® commercially prepared with fats, carbohydrates, proteins, vitamins, and minerals). Blood samples were collected at 0, 30, 60, 90, 120, 180, 240, and 360 min after EGCG intake. Free EGCG in plasma was measured using a liquid chromatography and mass spectrometry UPLC-ESI-MS/MS analytical method. The pharmacokinetic variables analyzed statistically were area under the curve ($AUC_{0–360}$), C_{max}, C_{av}, C_{min}, $T_{1/2}$, and T_{max}. EGCG (Teavigo®) alone was the group with higher $AUC_{0–360}$, C_{max}, and C_{av} both in men (3.86 ± 4.11 μg/mL/kg/6 h; 5.95 ng/mL/kg; 2.96 ng/mL/kg) and women (3.33 ± 1.08 μg/mL/kg/6 h; 6.66 ng/mL/kg; 3.66 ng/mL). Moreover, FontUp® was the group with the highest value of $T_{1/2}$ both in men (192 ± 66 min) and women (133 ± 28 min). Teavigo® intake after fasting overnight revealed the highest concentration of EGCG in plasma according to its pharmacokinetic profile, indicating that this is an excellent alternative of administration if the

experimental design requires good absorption in the gastrointestinal tract. Moreover, EGCG taken along with food supplements (FontUp®) improved the stability of the molecule in the body, being the best choice if the experimental design wants to reduce interindividual variation.

Keywords: epigallocatechin gallate; EGCG; catechins; polyphenols; green tea; Teavigo®, bioavailability; pharmacokinetic profile; antioxidants; food supplement

1. Introduction

Tea is one of the most popular beverages consumed across the world. It is extracted from the unfermented leaves of *Camellia sinensis* and mainly produced in four varieties, white, green, oolong, and black, depending on the oxidation and fermentation techniques applied [1]. Daily intake of green tea provides several health benefits, such as anti-inflammatory, anticarcinogenic, antimicrobial, and antioxidant effects reducing the risk of various diseases [2]. The health benefits of green tea are mainly attributed to its antioxidant properties [3]. For that reason, green tea extracts have been evaluated in diseases associated with an increase of reactive oxygen species (ROS) and oxidative stress, such as cancer and cardiovascular diseases [4,5]. Moreover other molecular mechanisms like signaling pathways, the modulation of some enzyme activities, and several interactions with membrane receptors related to cognitive functioning and Alzheimer's disease have also been associated to green tea components [6,7].

Most of the health-promoting effects of green tea are associated to its polyphenol content [8], particularly flavonoids. The main flavonoids in green tea, the catechins, make up to 30%–40% of the solid components of green tea. The major catechins in tea are epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG). EGCG, the most abundant flavanol, represents approximately 59% of the total catechins [2]. Many of the beneficial properties of green tea are attributed to this compound so that, recently, EGCG has been raised as a potential therapeutic tool [9,10]. Some of these health effects are related to the antiproliferative role of EGCG by the interference of the intracellular signaling cascades, which inhibits cell growth at the G1 stage, triggering apoptosis. In this framework, EGCG has been proposed as a chemopreventative in cancer prophylaxis [11,12]. Other beneficial properties of EGCG are its metabolic effects reducing the risk of type 2 diabetes and its cardiovascular complications [13]; antimicrobial activity due to the damage produced in the bacterial cell membrane when catechins bind to the lipid bilayer, inhibiting the ability of the bacteria to bind to the host cells; and its role in prevention and reduction of viral infections. EGCG also exercises a protective role in neurodegenerative diseases like Alzheimer disease [2] or after a neural injury [14]. Additionally, a recent research has demonstrated that this flavonoid improves cognitive performance and adaptive functionality in individuals diagnosed with Down syndrome by modulating the overexpression of the dual specificity tyrosine phosphorylation regulated kinase 1A (Dyrk1A) [15]. This protein is encoded by the *DYRK1A* gene, involved in signaling pathways which regulate cell proliferation, neural plasticity, and neurogenesis [16].

EGCG disposition depends on the ADME (absorption, distribution, metabolism, and excretion) processes, reaching the plasmatic peak concentration at 90 min and being undetectable 24 h after its oral intake [17]. EGCG reaches the stomach after oral administration, where the acid pH favors the structural stability of this molecule [18]. Then, a fraction of EGCG is absorbed in the small intestine. However, the low concentrations of EGCG observed in peripheral blood are caused by the transit of a substantial fraction of EGCG from the small to large intestines, where this molecule is transformed by the enterocytes and catabolized by local microbiota, leading to the formation of up to eleven catechin ring-fission products in different species. The EGCG ring-fission metabolites produced by intestinal microbiota are present as free and conjugated forms in plasma. In vitro data suggested that EGCG forms could reach the brain parenchyma by crossing the blood–brain barrier (BBB). Once distributed

throughout the brain, EGCG promotes neuritogenesis, showing an important role in suppressing neurodegenerative diseases [19]. Finally, the liver cells metabolize the remaining EGCG, transforming into methylated, sulfated, and glucuronide intermediates which will be further eliminated in urine [20].

Moreover, several factors affect to the stability of polyphenols and promote remarkable differences in the pharmacokinetic parameters of EGCG among individuals [17,21]. The concentration of EGCG is reduced by high pH (4 or higher) and high temperatures (20 °C or higher), which directly affect its structural stability, promoting the chemical degradation of EGCG [22]. Recent studies postulate that the oral bioavailability of EGCG is low in humans and decreases if accompanied by food [17,23]. In contrast, others studies conclude that the intake of EGCG with some specific nutrients such as fish oil (omega-3 fatty acids) [24], vitamins as ascorbic acid which reduce the oxidation of EGCG [25], and minerals as selenium or chrome [25,26] improves the EGCG bioavailability, enhancing its antioxidant activity.

Due to its hydrophilic nature, EGCGs show homogeneity problems in lipid products, affecting not only its appearance but also its effectiveness. Structural modifications of EGCG via esterification with aliphatic molecules such as long-chain fatty acids have been performed to solve these problems, and they can serve as a useful tool in altering its physical properties [27]. Interestingly, these ester derivatives have shown higher antiviral and antioxidant properties than the parent EGCG molecule and enhance its cellular absorption in vivo [27–29]. Moreover, EGCG derivates were demonstrated to be more effective in neuroprotection that non-modified EGCG in a cellular model of Parkinson [30]. Thus, EGCG esters may be used in nutrition and cosmetics as lipophilic alternatives with no effects in its functional properties.

Human studies evaluating the bioavailability of green tea extracts purified up to 95% in EGCG in different conditions (i.e., after overnight fasting, with additional nutrients, or included into food supplements) are scarce [31]. For that reason, the pharmacokinetic profile of EGCG in humans as well as its bioavailability is still investigated [21,32,33]. Different studies in both animal models and humans show controversial results regarding the bioavailability and also the biological effects elicited by EGCG [31,34]. Teavigo® capsules are a purified green tea extract with an EGCG content around 94% for which solubility and stability have been improved from previous products containing green tea extracts. Teavigo® is also incorporated into nutritional supplements as FontUp®, which also contains vitamins (A, C, D, E, K, and B1), minerals (zinc, chrome, selenium, etc.), and fatty acids as omega-3 in order to get better stability and to reduce the potential degradation of the molecule after dilution in various liquids such as milk and water [35]. Therefore, Teavigo® is frequently used in clinical trials because it has enabled the use of EGCG with very minor contributions of other green tea catechins. In clinical trials, EGCG concentrations range from 100 mg to 600 mg per day [36]. A daily ration of nutritional supplement usually contains between 200 to 400 mg of Teavigo®. Doses higher than 800 mg per day have shown toxic effects in cellular and animal models with no additional benefits [37]. The European Food Safety Agency (EFSA) reviewed recently the safety of EGCG and concluded that there is evidence from interventional clinical trials that intake of doses equal or above 800 mg EGCG/day taken as a food supplement have been shown to induce a statistically significant increase of serum transaminases in treated subjects compared to controls. Other side effects described in this report include dizziness, anemia, hypoglycemia, and kidney problems [38].

A randomized and crossover study was performed in order to evaluate the bioavailability of EGCG in healthy subjects. EGCG concentrations in plasma samples of healthy male and female volunteers were analyzed after the administration of green tea extract Teavigo® in three different ways: (1) fasting conditions, (2) combined with a Mediterranean diet breakfast, and (3) in the form of the commercial dietary supplement Fontup®. Thus, our results will determine the effect of different nutrients on the absorption and the stability of EGCG.

2. Materials and Methods

2.1. Participants and Selection Criteria

Twenty-two Caucasian healthy adult volunteers were prospectively recruited in the study. All participants were physically examined before the start of the study to confirm their healthy condition. In the first clinic visit, the anthropometric parameters, gender, age, weight, and height, were collected and body mass index (BMI) was calculated for all individuals.

Exclusion criteria for subjects were a BMI of ≤18 kg/m^2 or ≥30 kg/m^2; smoking; pregnancy; use of oral contraceptives; any intake of medication; functional food or dietary supplement (including green tea); and any known renal, hepatic, gastrointestinal, hematological, endocrinological, pulmonary, cardiovascular, or malignant disease. Eight volunteers met exclusion criteria. The remaining fourteen subjects were included in the study and randomized by gender under equal conditions.

Sample size was calculated using a two-sided contrast method with a significance level of $\alpha = 0.05\%$ and a statistical power of 80% ($\beta = 0.2$), assuming 10% of missing values. An accuracy value of 40 and a variance of 1000 in the control group were estimated based on blood EGCG values published previously [39]. Finally ten participants, five men and five women aged between 25 and 35 years of age, were selected and included in the study, discarding four participants not included in this age range [17,40,41].

Written informed consent was obtained from all the participants before the start of the study. All the protocols performed in this study were approved by the local ethical committee (CEIC-FSJD: Comitè Ètic d'Investigació Clínica—Fundació Sant Joan de Déu, ref. 2018/PIC-11-18) and were conducted according to the Declaration of Helsinki principles. All methods performed in this study were in accordance with the relevant guidelines and regulations.

2.2. Reagents and EGCG Preparations

Teavigo® (94% EGCG, 150 mg of green tea extract, 60 capsules) was provided by Healthy Origins (Pittsburgh, USA). The food supplement FontUp® (94% EGCG, 266 mg of concentrated green tea extract plus fats, carbohydrates, proteins, vitamins, and minerals in format sachet; see Table A1) was purchased from Grand Fontaine Laboratories (Barcelona, Spain). The concentration (purity) of the EGCG of each product was stated by the manufacturers.

Acetonitrile high-performance liquid chromatography (HPLC-grade), methanol (HPLC-grade), acetone (HPLC-grade), and glacial acetic acid (99.8%) of analytical grade (Scharlab, Barcelona, Spain) were used. Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Standards of EGCG and ethyl gallate (internal standard, I.S.), ascorbic acid (A92902) and Ethylenediaminetetraacetic acid (EDTA, E9884) were purchased from Sigma Aldrich (St. Louis, MO, USA). EGCG-free plasma for ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis was obtained from the Hospital del Mar blood bank (Barcelona, Spain). Monosodium dihydrogen orthophosphate (NaH_2PO_4:H_2O) was purchased from Merck. All food ingredients for breakfast were purchased from local supermarkets.

2.3. Interventions

The clinical design included the administration of Teavigo®, Teavigo® plus breakfast, and FontUp® intake, with a washout period of seven days between interventions. Before starting the crossover study, the sequence of interventions was randomized for each subject. In all cases, the administration was performed after a fasting period of at least 8 h. No subject dropped out of the study.

For Teavigo® series, the volunteers ingested 300 mg of green tea extract (two capsules with 140 mg of EGCG) with 100 mL of water. FontUp® is a nutritional supplement with a cocoa taste formulated to provide the nutrients contained in a complete diet (188 kcal per ration, see Table A1) considering several comorbidities of subjects having Down syndrome (celiac disease, gluten intolerance, gastrointestinal disturbances, etc.). FontUp® (266 mg of green tea extract, minimum 250 mg of EGCG) was dissolved

into 200 mL of semi-skimmed milk (200 kcal) and administered with no additional breakfast. Moreover, in the Teavigo® plus breakfast series, two capsules of Teavigo® were administered with 30 g of breakfast cereals, 200 mL of semi-skimmed milk with soluble cocoa, and a couple of toasts with 5 mL olive oil, resulting in 480 kcal of caloric intake (Table 1). EGCG preparations were administered to all subjects within 5 min, and no additional food was taken for 6 h.

Table 1. Nutrients of the standardized breakfast included in the Teavigo® plus breakfast intervention.

Nutrient	Weight (g)	% (*w*/*w*) [1]	kcal
Semi-skimmed milk + Soluble cocoa	200	78.4	200
Breakfast cereals	30	11.8	120
2 bread toasts + 5 mL Olive oil	25	9.8	160
Total	**255**	**100**	**480**

[1] w = weight.

2.4. Blood Collection, Processing, and Plasma Storage Protocol

An 18-gauge shielded IV catheter with injection port (1.3 × 33 mm) and three-way stopcock extension (Vasofix® Safety, Braun, Kronberg, Germany) for serial extractions was placed in the median cubital vein of the antecubital fossa for all the participants and was left in for 6 h.

Five mL of whole blood was collected into lithium heparin tubes (BD Biosciences, Madrid, Spain) before the intervention and at 30, 60, 90, 120, 180, 240, and 360 min after the ingestion of each EGCG preparation. Immediately after collection, the blood samples were maintained on ice, and within 10 min of blood collection, plasma was separated by centrifugation for 10 min at 1750× *g* at 4 °C. Plasma samples (0.350 mL) were stored in Eppendorf low binding tubes (Sarstedt 72.706.600, Nümbrecht, Germany) containing 20 µL of a preserving solution (1.38 g $NaH_2PO_4 \cdot H_2O$, 5 g of ascorbic acid, and 25 mg EDTA in 25 mL milli-Q H_2O, pH 3.8) and kept at −80 °C until further analysis.

2.5. Determination of Free EGCG in Plasma Samples

2.5.1. Preparation of Standard Solutions

Standard EGCG solutions (in methanol at 0.1% HCOOH) were prepared the day before each analytical batch and stored in a dark-glass flask at −18°C to prevent degradation. Working solutions of 0.1, 1, and 10 µg/mL were used to prepare calibration curves each analysis day, which consisted of at least two replicas at seven different concentrations (10, 29, 43, 71, 100, 286, and 429 ng/mL).

2.5.2. Sample Preparation

The evaluation of free EGCG concentration in plasma samples was performed by an extraction procedure described by Martí et al. (2010) with some modifications [42]. Briefly, a solid-phase extraction was done in OASIS hydrophilic-lipophilic-balanced (HLB) µElution Plates 30 mm (Waters, Milford, MA, USA) that were conditioned sequentially with 250 µL of methanol and 250 µL of 0.2% acetic acid. Before loading to the plate, plasma samples were mixed with 350 µL of phosphoric acid 4% and centrifuged for 10 min at 4 °C and 16,000× *g*. Later, plates were washed with 200 µL of Milli-Q water and 200 µL of 0.2% acetic acid, and finally, samples were eluted with 100 µL of acetone/acetic acid solution 2% (70:30, *v*/*v*). Five µL of the eluted solution was directly injected in the UPLC-MS/MS.

2.5.3. UPLC-ESI-MS/MS

The analytical quantification was performed using an Acquity UPLC system (Waters Associates, Milford, MA, USA) coupled to a triple quadrupole (QuattroPremier, Waters, Milford, USA) mass spectrometer provided with an orthogonal Z-spray-electrospray interface (ESI) (Waters Associates). The chromatographic separation was performed at 55 °C using an Acquity UPLC BEH C18 (100 mm ×

3.0 mm i.d., 1.7 µm) column at 0.3 mL/min of flow rate. The mobile phase consisted of formic acid 0.1% (A) and acetonitrile with formic acid 0.1% (B), with the following gradient program: from 99.5% B (maintained 1 min.) to 50% B in 1 min. After 2 min, the gradient was back to initial conditions. Total time of chromatogram was 6 min.

The mass spectrometry (MS) used nitrogen as drying and nebulizing gas. The desolvation gas flow was set at 1200 L/h, and the cone gas flow was set at 50 L/h. The selected capillary voltage was 0.4 kV in negative ionization mode. The nitrogen desolvation temperature was 450 °C, and the source temperature was set to 120 °C. The collision gas was argon at a flow of 0.21 mL/min. The detection of the analytes was performed by the selected reaction monitoring (SRM) method. Mass/charge values selected for identification of analytes were as follows: EGCG *m*/*z* 457 → 139, 169, and 305; collision energy (CE) 15 eV for all the transitions; and ethyl gallate, *m*/*z* 197 → 78, 124, and 169 and CE 30, 10, and 10 eV, respectively.

2.6. Statistical Analysis

Database management and statistical analysis of the pharmacokinetic variables and the anthropometric measurements were performed using SPSS v.22 (IBM, Armonk, NY, USA) and GraphPad software 6.0 (Prism, San Diego, USA). Descriptive statistics were performed using mean, Standard Deviation (SD), and error (Std. Error). T-test was used to compare the distribution of age and gender for the different groups, using the Holm–Sidak correction. The one-way ANOVA and the Bonferroni post hoc tests were used to determine differences between the mean values of the anthropometric and pharmacokinetic variables obtained for the three EGCG series. Statistical significance was set at $p < 0.05$ for all the analyses performed. All pharmacokinetic data were calculated and presented in accordance with internationally accepted and standardized methods [43,44]. The correlations were also analyzed using linear regression analysis (SPSS v.22). Pharmacokinetic analysis was performed in accordance with current industry guidance for orally administered pharmaceutical products [44].

The maximum concentration of EGCG from time 0 to 6 h was defined as C_{max}, with T_{max} being the time required to reach the C_{max}. The concentration of plasma EGCG at the end of the dosing interval was defined as C_{min}, and the mean concentration during the dosing interval was defined as C_{av}. The plasma EGCG elimination half-life ($T_{1/2}$) was calculated based on the formula $T_{1/2} = 0.693/Ke$, where Ke is the slope of the logarithmically transformed (ln) linear regression of the plasma EGCG concentrations. The area under the curve ($AUC_{0–360}$) analysis was determined using the linear trapezoidal rule from 0–6 h.

3. Results

3.1. Anthropometric Data of Participants and EGCG Administration

Five healthy men and women were selected from the initial twenty-two volunteers to evaluate the EGCG concentrations in plasma (Figure 1). Sample size was calculated with a significance level of $\alpha = 0.05\%$ and $\beta = 0.2$, obtaining a minimum value of ten subjects to get statistical significance. Previous studies on green tea or EGCG in volunteers were performed with a similar sample size [17,40,41]. The anthropometric data of all participants (age, height, weight, and BMI averages distributed by gender) were measured at baseline of the clinical trial (Figure 1, details in Table 2). Statistical analysis (*t*-test) demonstrated that there was no significant difference in age (*p*-value = 0.57) between men and women. The age average for all participants recruited for this study was 29.7 ± 4. By contrast, height, weight, and BMI data showed expected differences related to gender. For men, averages were height, 176 ± 5 cm; weight, 73.8 ± 11.6 kg; and BMI, 23.7 ± 2.8 (kg/m^2). For women, the averages obtained were height, 163 ± 6 cm; weight, 52.8 ± 7.0 kg; and BMI, 19.9 ± 2.1 (kg/m^2).

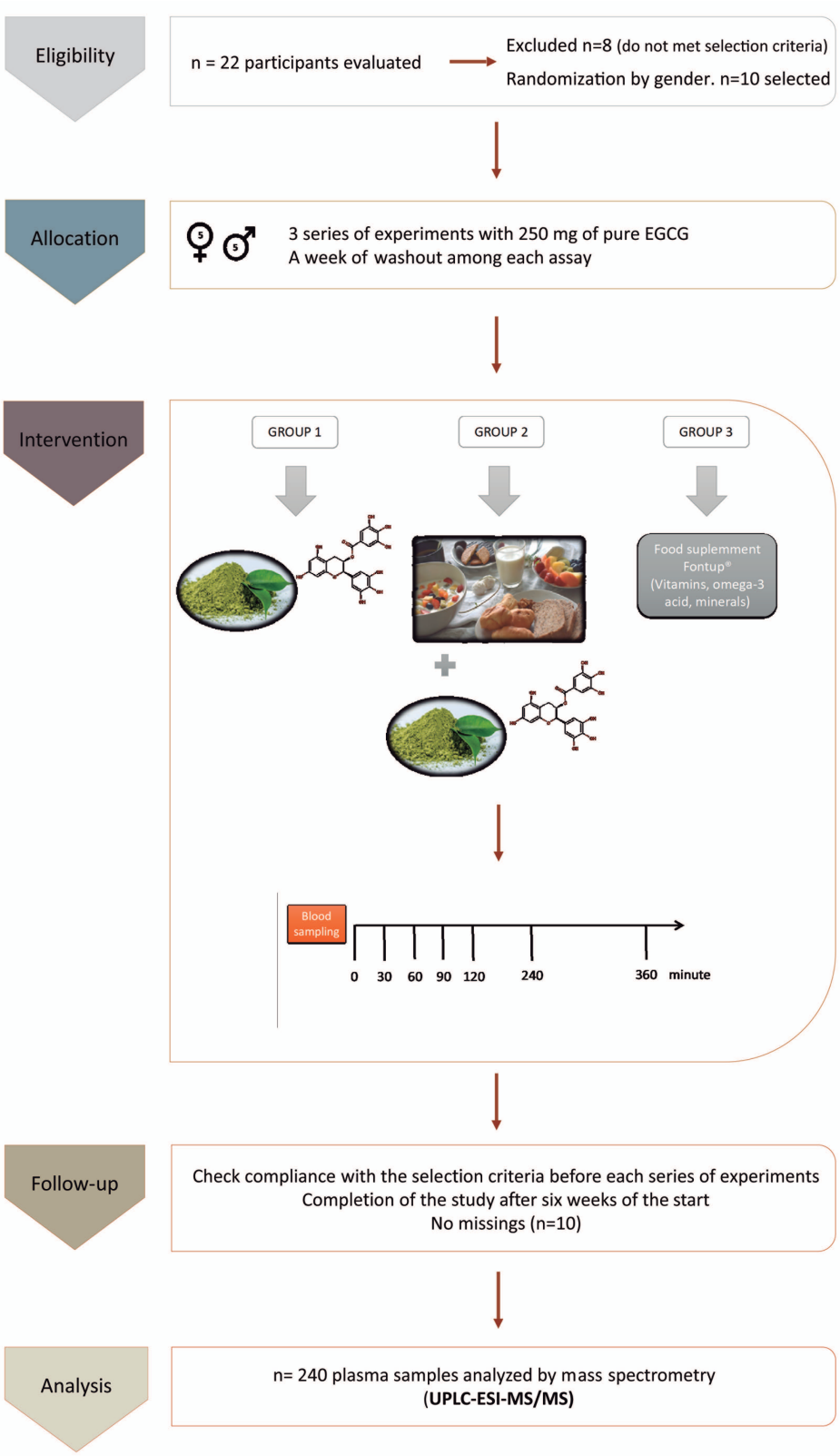

Figure 1. Consort flow chart and schematic representation of the study design and the blood sampling: The experimental design includes the study groups, the timeline of blood extractions after the intake of epigallocatechin-3-gallate (EGCG), and the methodology to analyze the samples. UPLC-ESI-MS/MS = ultra-performance liquid chromatography-electrospray tandem mass spectrometry.

Three series of EGCG delivery randomized for each subject at the beginning of the trial were performed as follows (Figure 1): Teavigo® in fasting conditions (without breakfast), administrating two capsules of Teavigo® with a standard breakfast (480 kcal, see Table 1), and finally a sachet of FontUp® (188 kcal, see Table A1) with semi-skimmed milk (200 kcal) containing at least 250 mg of EGCG. The nutrients included in the breakfast and their kcal are shown in Table 1. All experiments started after at least eight hours of overnight fast.

The selected dosage (300 mg of EGCG) has been previously tested in clinical trials for Down Syndrome [15,45], cardiovascular disease [46], and cancer [47,48], obtaining satisfactory results. In spite of the fact that the daily dose of EGCG can reach 800 mg per day, high doses are frequently administered two times per day: in the morning after overnight fasting and in the evening. Our experimental design has focused on the bioavailability of EGCG in the first daily dose in the morning used in clinical trials (between 200 and 400 mg of EGCG) performing different controlled conditions. Teavigo® intake with no additional nutrients was used as the ideal condition for EGCG administration with the least interference from other environmental or nutritional factors.

Table 2. Anthropometric data of the participants included in the study.

Demographic Parameters	Total	Men	Women
Nº of participants	10	5	5
Age mean (years)	29.7 ± 4.3	30.6 ± 5.2	28.8 ± 3.6
Height (cm)	169 ± 8.8	176 ± 5.0	163 ± 6.0
Weight (kg)	63.2 ± 14.4	73.8 ± 11.6	52.8 ± 7.0
BMI [1] (kg/m^2)	21.8 ± 3.1	23.7 ± 2.8	19.9 ± 2.1

[1] BMI = body mass index.

3.2. EGCG Concentration–Time Profiles in Plasma

The plasma EGCG concentrations were measured using ultra-performance liquid chromatography-electrospray tandem mass spectrometry (UPLC-ESI-MS/MS). This methodology has been previously validated [23,42]. Figure 2 shows analytical chromatograms of control (plasma at baseline) (Figure 2a), a blank spiked with a known concentration of an analytical standard of EGCG (Figure 2b), and a real sample (Figure 2c). No interfering peaks at the retention time of the EGCG peak were observed.

The EGCG plasma concentrations over time considering the three interventions are shown in Figure 3 (individualized following gender) and in Figure 4 (mean values per gender). Concentration values were expressed as ng/mL of EGCG in plasma per kilogram of weight.

The delivery of EGCG in fasting conditions, taking two capsules of Teavigo® without breakfast, showed higher concentrations of EGCG in plasma (C_{max} 5.9 for men and 6.7 ng/mL/kg for women) than Teavigo® plus breakfast (C_{max} 3.9 for men and 4.5 ng/mL/kg for women) and five-folds higher than the administration of the food supplement FontUp® containing EGCG (C_{max} 0.9 for men and 1.3 ng/mL/kg for women), being this difference statistically significant (p-value = 0.009 for men and 0.006 for women) (Figure 4, Table 3). Minimum concentrations, C_{min}, after six hours of EGCG intake did not result in statistical differences between Teavigo® and Teavigo® plus breakfast. In contrast, Teavigo® versus FontUp® showed significant differences (Table 3). Interestingly, a slightly and nonsignificant trend was observed when differences between men and women were analyzed for C_{max} and C_{min}, observing that the values of EGCG for all women included in this study were always higher than men (Figure A1). Moreover, the concentration–time curves showed a high variability among participants for Teavigo® without breakfast with non-dependency by gender (Figures 3 and 4, Table 3). This variability for EGCG concentration was also observed in the delivery of Teavigo® plus breakfast. By contrast, the administration of FontUp® generated more predictable concentrations in both men and women than the other treatments (Figures 3 and 4, Table 3, and Table A2).

Table 3. Plasma kinetic parameters for EGCG after the three preparations.

Parameters	G	Teavigo®	Teavigo® with Breakfast	FontUp®
$AUC_{0–360}$ (μg/mL/kg/6 h)	♂	3.9 ± 4.1	1.5 ± 0.6	0.6 ± 0.1 **
	♀	3.3 ± 1.1	2.4 ± 1.1	0.8 ± 0.2 **
C_{max} (ng/mL/kg)	♂	5.9 ± 4.1	3.9 ± 1.3	0.9 ± 0.1 **
	♀	6.7 ± 1.7	4.5 ± 2.1	1.3 ± 0.3 **
C_{av} (ng/mL/kg)	♂	3.0 ± 2.6	1.5 ± 1.5	0.6 ± 0.2 **
	♀	3.7 ± 2.3	2.1 ± 2.0	0.9 ± 0.4 **
C_{min} (ng/mL/kg)	♂	1.5 ± 1.4	0.7 ± 0.4	0.3 ± 0.1 *
	♀	2.2 ± 1.1	2.2 ± 1.6	0.3 ± 0.0 *
$T_{1/2}$ (min)	♂	154.2 ± 27.9	93.1 ± 36.2 *	191.7 ± 66.4
	♀	117.2 ± 53.5	111.4 ± 39.1	132.9 ± 27.9
T_{max} (min)	♂	120 (90–180)	120 (90–180)	90 (60–90)
	♀	90 (60–120)	180 (120–360)	120 (90–120)

Values are means ± standard deviations for the 10 participants ($n = 10$), except for T_{max} (median with the minimum and maximum time observed). * Value is significantly different from the other values in the row at the level of $p < 0.05$. ** Value is significantly different from the other values in the row at the level of $p < 0.001$. Abbreviations: G = gender; min = minutes; h = hours; ♂ = man; ♀ = women; $AUC_{0–360}$ = area under the curve from 0 to 6 h; C_{max} = maximum concentration; C_{av} = average concentration; C_{min} = minimum concentration (at the end of the treatment); $T_{1/2}$ = half-life; T_{max} = time required to reach the maximal concentration.

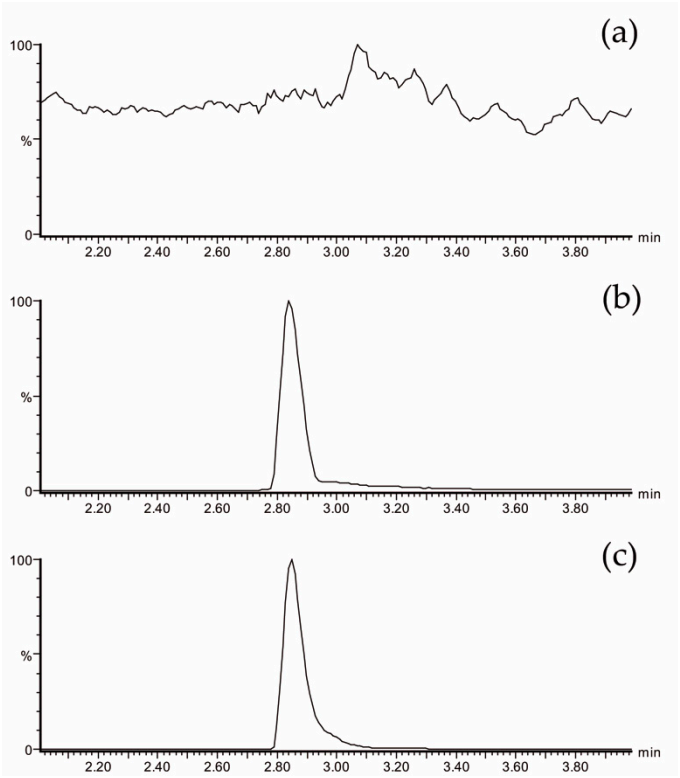

Figure 2. LC-MS/MS chromatogram of EGCG showing the most specific and sensitive MS/MS transition m/z 457→169: (**a**) plasma sample from a volunteer at baseline, (**b**) blank of plasma spiked with 428 ng/mL of EGCG, and (**c**) plasma sample from a volunteer at 60 min after Teavigo® ingestion (calculated concentration: 289 ng/mL). LC-MS/MS = liquid chromatography tandem mass spectrometry.

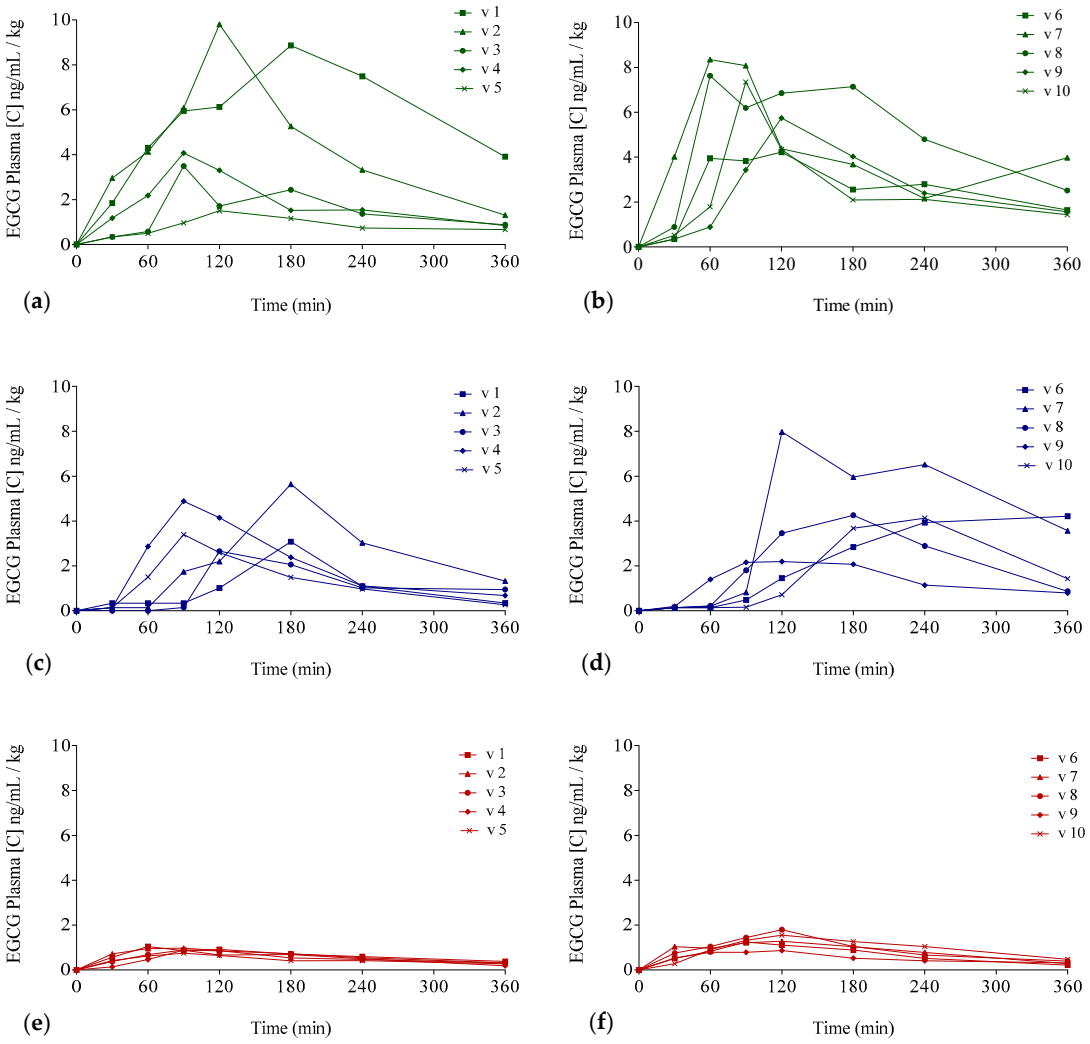

Figure 3. Plasma EGCG concentration–time curves for each of the participants for the three different EGCG preparations: Concentrations for men (**a**,**c**,**e**) and (**b**,**d**,**f**) for women. Teavigo® condition is shown by green lines (**a**,**b**), Teavigo® plus breakfast is shown by blue lines (**c**,**d**), and FontUp® is shown by red lines (**e**,**f**).

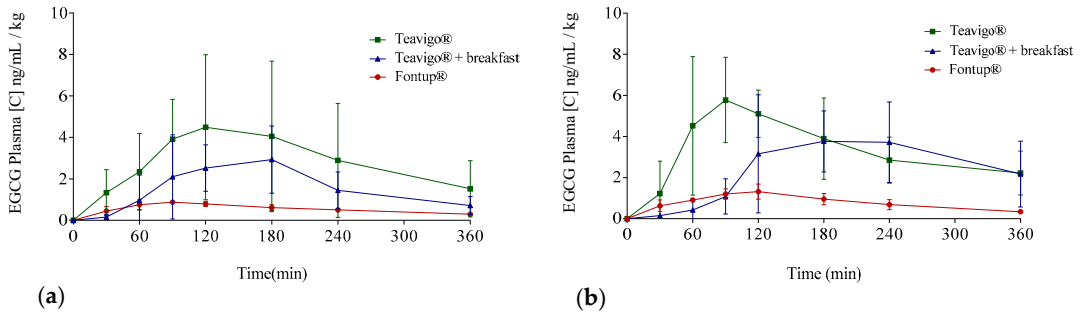

Figure 4. Averages of EGCG plasma concentrations over time distributed following EGCG preparation and gender: Teavigo® condition is shown in green lines, Teavigo® plus breakfast is in blue lines, and FontUp® is in red lines. (**a**) EGCG average concentrations for men and (**b**) EGCG average concentrations for women. Error bars represent the standard deviation.

3.3. Pharmacokinetic Parameters of EGCG in Plasma

The resulting values of the EGCG preparations showed differences by gender in the EGCG bioavailability (Figure 4). The peak of EGCG concentrations in plasma for Teavigo® plus breakfast and FontUp® intake reached later in women, with the T_{max} at 180 and 120 min respectively, than in men with the EGCG peak at 120 and 90 min. Conversely, the values of T_{max} for Teavigo® administration were 120 min in men and 90 min in women (Table 3). These differences were analyzed by statistically using one-way ANOVA corrected by the Tukey test for multiple comparisons. Interestingly, only Teavigo® plus breakfast versus Teavigo® in women had statistical significance (p-value = 0.020).

Following a single-compartment modeling, the mean half-life averages ($T_{1/2}$) were for men and women, respectively, Teavigo®, 154.0 ± 27.9 and 117.2 ± 53.5 min; Teavigo® plus breakfast, 93.1 ± 36.2 and 111.4 ± 39.1 min; and FontUp®, 191.7 ± 66.4 and 132.9 ± 27.9. $T_{1/2}$ showed statistical significance (p-value = 0.020) for Teavigo® versus FontUp® administration in men. These results highlight EGCG administration as FontUp® reaches the longest half-life in the body, indicating that EGCG is more stable when is accompanied by vitamins (A, C, D, E, and K), folic acid, the omega-3 fatty acid, and the minerals present in FontUp® than EGCG administered on an empty stomach or with a standard breakfast (Figures 4 and 5).

Statistical differences were also observed when C_{av} (ng/mL/kg) was analyzed in Teavigo® versus FontUp® preparations (Table 3, p-value = 0.025 for man and 0.005 for women). However, no statistical significance was observed between Teavigo® and Teavigo® plus breakfast for C_{av} (p-value = 0.34 for men and 0.23 for women, respectively). Teavigo® administration showed a C_{av} of 3.0 ng/mL/kg for men and 3.7 ng/mL/kg for women, while Teavigo® plus breakfast results were 1.5 ng/mL/kg for men and 2.1 ng/mL/kg for women. Additionally, the average concentration of EGCG contained in FontUp® reached 0.6 ng/mL/kg for men and 0.9 ng/mL/kg for women. Considering that the values for all volunteers were corrected by their own body weight, these results showed nonsignificant differences by gender despite the amount of EGCG detected in the circulatory system being higher in women than men for all preparations tested (see Figure A1).

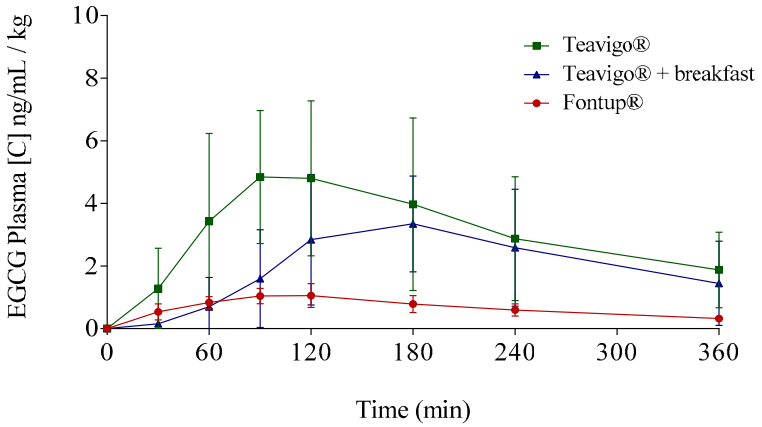

Figure 5. Mean plasma concentration time profiles of EGCG for the three different EGCG interventions by the oral route: Green line represents Teavigo®, blue line represents Teavigo® plus breakfast, and red line represents FontUp®. Each value was obtained by HPLC-MS analysis. Error bars represent the standard deviation.

One-way ANOVA analyses with Tukey post hoc test for multiple comparisons were performed to evaluate $AUC_{0–360}$ between interventions. $AUC_{0–360}$ for EGCG taken as Teavigo® capsules in fasting conditions (Table 3) was significantly higher than the $AUC_{0–360}$ for EGCG delivery with FontUp® (p-value = 0.036 for men and 0.001 for women). Moreover, the difference between the $AUC_{0–360}$ for Teavigo® with breakfast and the $AUC_{0–360}$ for EGCG present in FontUp® was also statistically significant (p-value = 0.030 for men and 0.023 for women) (Table 3). These results indicated that EGCG

delivery in FontUp® supplement is the least efficient way to absorb EGCG to the body, compared with Teavigo® with or without breakfast.

4. Discussion

In this study, we corroborated previous results regarding EGCG bioavailability depending on its ingestion with or without food [33]. The present study shows a higher bioavailability for the Teavigo® intake with no additional food compared to the other two preparations tested taken either with food or with a dietetic supplement. The increased global bioavailability of Teavigo® without food shows also a higher interindividual variability (up to 100% in males, based on AUC values). The FontUp® product displays the lowest bioavailability, less variability, and higher stability on EGCG plasma concentrations.

EGCG and other green tea polyphenols may have potential therapeutic applications mainly in the prevention of a large variety of human diseases. A large number of clinical studies have demonstrated the benefits of EGCG in patients diagnosed with cancer [49], neurodegenerative diseases [50], Down syndrome [51], and metabolic syndrome [52] by regulating various metabolic, genetic, and epigenetic pathways [53,54]. Several studies have shown important differences regarding the bioavailability of this molecule due to the heterogeneity of the human populations analyzed [21,55] and the differences in stability depending on the delivery strategy [23,35,39]. Additionally, the absorption and stability of EGCG are directly influenced by the combined intake of this molecule with other food products, which determine the environment of EGCG before its absorption and modulates its biological response. Unfortunately, the interactions of specific nutrients with the metabolic processes related to EGCG bioavailability remain unclear [23,31].

EGCG has been tested for therapeutic purposes in a range of doses from 150 to 400 mg per day, being the oral administration the most widely used delivery method in animal models and clinical trials in humans [31,45,56]. The blood concentrations obtained in this study for Teavigo® alone or with a Mediterranean diet breakfast (Tables 3 and A2) are consistent with previous works which analyzed the bioavailability of EGCG in a limited number of volunteers [23,36,55] or in a specific condition [21,39]. The present study moves one step forward using different nutritional strategies to deliver EGCG as well as a cohort of volunteers with an adequate sample size to analyze the EGCG dose used in clinical trials [15], taking also into account the gender balance. The objective is to determine the most appropriate conditions for EGCG intake.

EGCG has been administered in the form of green tea extract (Teavigo®), in a single dose of 250 mg after overnight fasting, showing significant differences according to the conditions and nutritional supplements used [23]. The intake of Teavigo® after fasting overnight results in the highest peak concentrations (C_{max}) and $AUC_{0–360}$ values in both genders (Figure 5). The comparison between Teavigo® and Teavigo® plus breakfast for each participant indicates that a Mediterranean diet breakfast reduces the bioavailability of EGCG (more than 100% in males and 30% in females following AUCs). For that, the green tea extract should be ingested alone after overnight fasting to optimize the gastrointestinal absorption of the EGCG. These results are consistent with previous studies in which the authors proposed that the administration of EGCG alone elicits an attenuated strong response from the stomach and pancreas, minimizing the digestion processes [17,57]. When a capsule containing EGCG with no additional food is ingested after fasting overnight and arrives to the stomach, the gastrointestinal processes are only partially activated just by that small amount of nutrients. Then, the EGCG molecules remain stable due to the propitious acidic environment (pH < 3), where the oxidation of their polar residues is minimized [58]. In addition, the neutralization process by the secretion of bile salts is not activated, which favors and increases their absorption by the enterocytes in the small intestine. Otherwise, the lack of activation of digestion mechanisms reduces the activity of the bacterial microbiota responsible for catabolizing these antioxidant molecules in the large intestine.

As mentioned before, a standard breakfast composed by milk, cocoa, cereals, and toasts with olive oil reduces the bioavailability of EGCG (Figure 5), in accordance with results obtained in other studies [23,35,39]. For example, some authors have concluded that the combined intake of Teavigo®

with semi-skimmed milk generates a significant decrease of EGCG bioavailability [23,35,39]. Moreover, Teavigo® taken with breakfast and, to a lesser extent, FontUp® showed a delay (T_{max}) to reach the higher concentration peak (C_{max}) of bioavailability in plasma (Table 3 and Figures 3–5). The intake of food products delays the rate of gastric emptying, which is critical to determine the absorption rate in the small intestine and influences the bioavailability of orally administered drugs [56]. More likely, the intake of enough amount of nutrients with EGCG activates the gastrointestinal processes and delays the gastric emptying. Consequently, the EGCG molecules spend more time on the basic environment of the small intestine, increasing their degradation. Therefore, the absorption of EGCG with additional nutrients is not only reduced but also delayed as compared to EGCG alone, which is able to pass directly to small intestine to be absorbed.

We also addressed the role of specific nutrients on the bioavailability of EGCG when those are ingested together with this molecule. In this line, the administration of the food supplement FontUp® has reported interesting results when it was compared with the intake of Teavigo® plus breakfast. It has been previously demonstrated that EGCG is unstable in environments with high temperature [59] and basic pH [60]. Accordingly, all the experiments have been performed using fresh fluids at 4 °C to reduce the degradation of EGCG, water in the case of Teavigo® alone, and semi-skimmed milk for the other two series of EGCG administration: FontUp® and Teavigo® plus breakfast. In reference to pH, when the food arrives to the small intestine, the pancreatic juice neutralizes the hydrochloric acid emptied into the duodenum from the stomach. Most likely, it is even more decisive that there is a lack of activation of gastrointestinal processes from the pH of the fluids and nutrients taken along with EGCG.

Moreover, ascorbic acid has been used in previous studies as a preservative to improve the bioavailability of EGCG by preventing oxidation at an acidic pH [25,57,61]. Vitamin C keeps the polarity of the eight hydroxyl groups and the structural stability of the molecule. The ratio used in FontUp® (12 mg of ascorbic acid per 250 mg of pure EGCG, see Table A1) reported the best stability parameters ($T_{1/2}$ =163 min) after gastrointestinal absorption compared to Teavigo® ($T_{1/2}$ =135 min) after fasting and Teavigo® plus a breakfast ($T_{1/2}$ =102 min). However, the presence of vitamin C in FontUp® did not improve the absorption of the molecule as did the presence of either sucrose previously described in literature [25]. Levels of EGCG in blood were 5-fold lower with FontUp® (C_{av} = 0.74 ng/mL/kg) compared to Teavigo® alone (C_{av} = 3.3 ng/mL/kg) and 2.5-fold lower than Teavigo® plus breakfast (C_{av} = 1.8 ng/mL/kg).

Otherwise, omega-3 polyunsaturated fatty acids from fish oil has been also tested to improve the intestinal absorption of EGCG [24,26,62]; however, its presence in FontUp® does not result in an improvement of the EGCG bioavailability compared to Teavigo® in fasting conditions or after a standard breakfast.

New delivery technologies to improve the oral bioavailability of EGCG have been raised to expand its application in a lipophilic media. Esterification of the water-soluble EGCG with stearic acid (SA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) or replacing the hydroxyls of EGCG with acetyl groups have demonstrated a significant improvement in stability, bioavailability, and bioactivities as antioxidants or antiproliferatives [27,30]. For that, their potential applications in future clinical studies should be analyzed in detail.

In reference to the limitations of our study, in spite of the sample size being calculated to obtain significant results in three series of experiments (n = 10), a higher number of volunteers would be desirable to reduce the interindividual variability related to EGCG disposition. Moreover, the main cause of interindividual variability is the low EGCG bioavailability estimated in humans (0.1%–0.3%) [63,64]. Small changes in this percentage result in significant changes in plasma concentrations beyond the differences among individuals in the genetic background related to EGCG metabolism. For example, a variation of EGCG bioavailability from 0.3% to 0.6% causes an increase of 100% in the EGCG plasma concentration. For these reasons, the present crossover study tries to reduce the impact of interindividual variation due to each volunteer used as its own control.

The oral administration of a green tea extract with a high percentage of EGCG (Teavigo®) has been unequivocally demonstrated as the proper form to obtain the higher bioavailability values. In contrast, EGCG accompanied by specific nutrients inside the food supplement FontUp® showed the most homogeneous disposition for all participants. Recently, it has been shown that FontUp® at doses within the range of those used in clinical studies normalized brain and plasma biomarkers deregulated in Dryk1a transgenic mice (TgBACDyrk1A), without negative effects on liver and cardiac functions [65]. Therefore, FontUp® or EGCG alone will be selected for clinical trials depending on the experimental design of the study, the goals, the variables to study, the environmental conditions, and other particular considerations.

5. Conclusions

EGCG is being evaluated as a promising compound for the treatment of human noncommunicable diseases such as cancer and cardiovascular, hepatic, and neurodegenerative diseases. However, the exposure to EGCG required for the treatment of each pathology is still under study. Additionally, it is well known that the EGCG bioavailability shows a high interindividual variability related to the gastrointestinal absorption, the stability of the molecule, the nutritional environment, and the administration conditions. The present research highlights that the use of a green tea extract enriched with EGCG (Teavigo®, 94% EGCG) after fasting overnight leads to the highest exposure to EGCG both in men and women ($AUC_{0–360}$ and C_{max}). Moreover, Teavigo® ingested with a Mediterranean diet breakfast shows a reduction in EGCG bioavailability. However, neither are the nutrients ingested at breakfast decisive in blocking the absorption of ECGC nor do the contents of the nutritional supplement FontUp® promote such absorption. In contrast, the supplements contained in FontUp® are able to favor the stability of EGCG in the gastrointestinal tract, and therefore, its use may be appropriate when the experimental design needs to reduce the interindividual variability and to analyze the efficacy of a stable and similar EGCG concentration in all study participants.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Food supplement FontUp® composition.

Average Analysis		Per 100 mL	Per Ration
Energetic value (kJ/kcal)		1606/383	787/188
Fats (g)		11	5.4
	Saturated (g)	4.4	2.2
	Monounsaturated (g)	4.4	2.2
	Polyunsaturated (g)	2.2	1.1
	Docosahexaenoic acid (DHA, mg)	5.4	2.6
Carbohydrates (g)		47	23
	Dietary fiber (g)	16	7.9
	Fructo-oligosaccharides (g)	3.1	1.5
Proteins (g)		16	8
Salt (g)		0.52	0.3
Minerals			
	Na (mg)	206	101
	K (mg)	606	297
	Cl (mg)	395	194
	Ca (mg)	221	108
	P (mg)	209	102
	Mg (mg)	106	52
	Fe (mg)	4.6	2.3
	Zn (mg)	3.1	1.5
	Cu (µg)	0.5	0.2
	Mn (mg)	0.5	0.2
	F (µg)	0.1	0.05
	Se (µg)	10	4.9
	Cr (µg)	16	7.8
	Mo (µg)	10	4.9
	I (µg)	21	10
Vitamins			
	Vitamin A (µg)	251	123
	Vitamin D (µg)	1.9	0.9
	Vitamin E (mg)	4.2	2.1
	Vitamin K (µg)	15.0	7.4
	Vitamin C (mg)	25	12
	Thiamine (B1) (mg)	0.3	0.1
	Riboflavin (B2) (mg)	0.4	0.2
	Niacin (B3/PP) (mg)	4.0	2.0
	Vitamin B6 (mg)	0.4	0.2
	Folic acid (B9) (µg)	80.0	39
	Vitamin B12 (µg)	0.5	0.2
	Biotin (µg)	58.0	28
	Pantothenic acid (B5) (mg)	1.9	0.9
Others			
	Green tea extract (mg) (minimum 250 mg EGCG)	543	266
	Osmolarity (mOsm/L)	635	

Table A2. Plasma kinetic parameters for EGCG after the three different series of experiments.

Parameters	N	Teavigo®	Teavigo® with Breakfast	FontUp®
$AUC_{0–360}$ (µg/mL/6 h)	♂	270.4 ± 288.2	108.6 ± 40.7	38.5 ± 5.8 **
	♀	233.2 ± 76.1	171.8 ± 77.7	54.7 ± 12.3 **
C_{max} (ng/mL)	♂	528.6 ± 472.3	275.3 ± 89.1	63.7 ± 7.7 **
	♀	466.1 ± 116.3	318.7 ± 147.0	94.5 ± 24.5 **
C_{av} (ng/mL)	♂	271.4 ± 299.2	108.4 ± 102.3	42.8 ± 16.6 **
	♀	256.0 ± 162.4	144.8 ± 143.2	60.4 ± 27.4 **
C_{min} (ng/mL)	♂	148.7 ± 188.1	50.1 ± 30.6	20.9 ± 4.8 *
	♀	155.8 ± 74.7	152.4 ± 112.0	23.8 ± 6.8 *
$T_{1/2}$ (min)	♂	154.2 ± 27.9	93.1 ± 36.2 *	191.7 ± 66.4
	♀	117.2 ± 53.5	111.4 ± 39.1	132.9 ± 27.8
T_{max} (min)	♂	120 (90–180)	120 (90–180)	90 (60–90)
	♀	90 (60–120)	180 (120–360)	120 (90–120)

Values are means ± standard deviations for the 10 participants ($n = 10$), except for T_{max} (median with the minimum and maximum time observed). * Value is significantly different from the other values in the row at the level of $p < 0.05$. ** Value is significantly different from the other values in the row at the level of $p < 0.001$. Abbreviations: G = gender; min = minutes; h = hours; ♂= man; ♀= women; $AUC_{0–360}$ = area under the curve from 0 to 6 h; C_{max} = maximum concentration; C_{av} = average concentration; C_{min} = minimum concentration (at the end of the treatment); $T_{1/2}$ = half-life; T_{max} = time required to reach the maximal concentration.

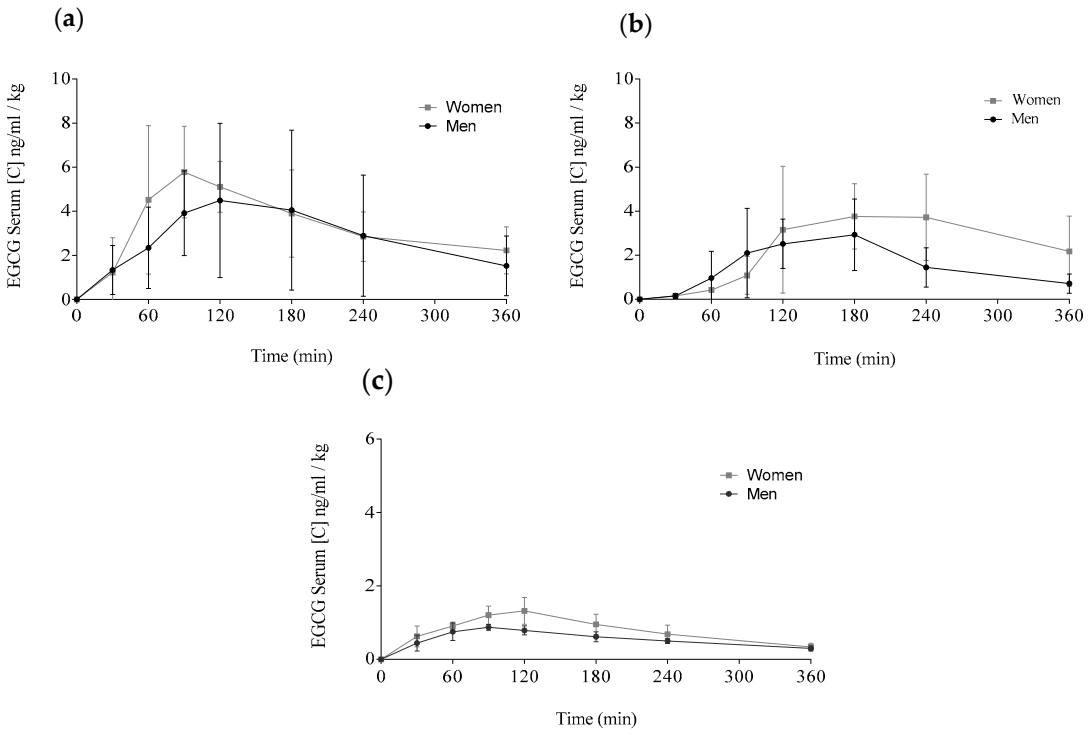

Figure A1. Comparison of the EGCG means distributed by delivery method and gender: Concentrations for women are showed by grey lines and, for men, by black lines. (**a**) Teavigo® alone, (**b**) Teavigo® plus breakfast, and (**c**) FontUp®. Each value was obtained by HPLC-MS analysis.

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DISCUSSION

5. DISCUSSION

Alcohol intake during pregnancy results in a phenotypic presentation of the unborn child characterized by fetal growth restriction, facial dysmorphologies, and CNS disorders4. FASD includes all diagnostic categories (FAS, pFAS, ARND, and ARBD), ranging from the complete syndrome (FAS) to partial phenotypes including documented or undocumented PAE (pFAS, ARND, and ARBD)[5]. PAE is an important preventable cause of intellectual disability[11–13] that poses significant health and economic burden for the society. Diagnostic difficulties, limited knowledge on the teratogenic effect of alcohol, and lack of effective therapies represent a major medical weakness.

The study of the underlying mechanisms of alcohol toxicity and evaluation of therapeutic strategies is possible with FASD-like animal models. Researchers must choose the animal model that best fits their experimental design and research question. Although invertebrates and simple vertebrates offer the possibility to study physical malformations and simple behaviors[293], mammals allow the study of brain structures and complex behaviors[26]. Murine models are widely used studying FASD, e.g., rodents for assessing dose-dependent alcohol teratogenic effects based on timing and developmental stage, human-like behaviors, or promising therapies. Moreover, mice and humans have similar neurodevelopmental stages, although the third trimester of human pregnancy is equivalent to the postnatal period in mice. Facial dysmorphology appears during the first trimester equivalent of alcohol exposure[44,69,294], but brain developmental processes occur continuously throughout the pregnancy. Thus, if PAE occurs at any time during pregnancy, key processes such as proliferation[57], migration[58], differentiation[59], synaptogenesis[60,61], gliogenesis, myelination[62], and apoptosis[63,64] are altered and may lead to brain and behavioral disorders in the fetus. Fetal growth is also impaired by the harmful effects of alcohol on angiogenesis. Fetal growth restriction may be evaluated through standardized fetal measurements in defined frequency distribution curves[76] or by assessing the placenta using biomarkers and histopathological analyses[212,213]. The ultimate goal of animal experimentation is to improve diagnostic strategies, mechanisms of prevention, and therapeutic options for FASD patients.

Other important variables to consider in animal experimentation are the pattern of ethanol exposure, alcohol dose, and route of administration. Some reports support the idea that lower daily doses of alcohol administered in a binge-like pattern result in worse neurological outcomes than higher daily doses delivered in a non-binge-like pattern, although binge-like patterns lead to higher BAC[35,295]. The route of administration directly affects variables such as the alcohol exposure pattern and amount of alcohol taken. Voluntary ethanol feeding and intragastric gavage are the most physiological methods,

but less time-efficient than inhalation[43] or injection[44]. Voluntary ethanol feeding is considered the safest technique, while intragastric gavage offers a more accurate control of the doses and timing, and higher BACs are reached compared to voluntary ethanol feeding[40–42]. In our experimental model, intragastric gavage was performed to ensure precise control of the dose and timing of ethanol exposure. We used continuous PAE during the first and second trimester equivalents simulating the two human drinking patterns (binge versus Mediterranean) to determine the effects of PAE on placental and fetal brain development.

As described in the introduction, multiple pathological effects are induced by PAE depending on the studied organ, region, or type of cell[216]. PAE teratogenic effects include damages caused by oxidative stress and other pathophysiology conditions as dysregulation of the neuroimmune system, neurotransmitter disorders, and epigenetic modifications. Experimental therapies to reduce oxidative stress and epigenetic alterations include the use of EGCG, a natural antioxidant extracted from green tea[249]. Additionally, EGCG improves neuronal plasticity in Down's syndrome patients by blocking Dyrk1A overexpression, a general inhibitor of neuronal plasticity[171]. In this thesis, we assessed the impact of EGCG treatment on oxidative stress, fetal growth, placental development, and neurogenesis in a FASD-like mouse model for two human-like drinking patterns (binge versus Mediterranean) during the first and the second trimester equivalents.

The findings reported in the first article of this thesis (Epigallocatechin Gallate Ameliorates the Effects of Prenatal Alcohol Exposure in a Fetal Alcohol Spectrum Disorder-Like Mouse Model), support EGCG as a promising antioxidant therapy to attenuate the consequences of prenatal alcohol exposure. Antioxidant therapy may ensure proper development of the placenta and fetal growth. Additionally, prenatal effects of EGCG on neural maturation and differentiation processes may lead to normal neurodevelopment, improving behavioral and cognitive outcomes in children.

The placenta ensures correct fetal development through the supply of nutrients and waste removal. Thus, normal invasion of uterine spiral arteries by trophoblasts is critical for the establishment of that interface. In this process, small muscular arteries becomes distended, flaccid, high flow-resistance vessels enabling nutrient exchange in the feto-maternal barrier[77]. PAE exerts a negative influence on placental development and fetal growth[77,296]. Our results show an inverse relationship between fetal growth and PAE, i.e., fetuses exposed to high doses of alcohol show lower fetal weights compared to controls. These results are in line with previous studies that report FGR in fetuses prenatally exposed to ethanol[296] and dose-dependent effects of PAE on fetal weight[77]. Angiogenic factors follow a dynamic expression pattern according to the metabolic requirements of

each gestational stage. Moreover, PAE leads to imbalances of the expression of these angiogenic factors with the consequent abnormal placental development[212,213] [34,35]. VEGF-A is a regulator of angiogenesis and vascular permeability. Results from this thesis show downregulation of VEGF-A in placentas from fetuses prenatally exposed to ethanol in both human-like drinking patterns. These findings support the deleterious effect on placental angiogenesis of any type of alcohol consumption during pregnancy. Similarly, PAE-related decreased levels of placental VEGF-A have previously been reported by other authors[213]. By inhibiting excessive proliferation of endothelial cells, VEGF-R1 acts a negative regulator of embryonic angiogenesis[297]. Our experiments indicate an increase in VEGF-R1 expression with continuous PAE. Upregulation of this antiangiogenic factor may be responsible for abnormal placental angiogenesis. Other authors reported discordances in VEGF-R1 expression depending on the timing of PAE. Our results are similar to those published by Ventureira et al. when PAE occurs until GD10[213]; on the other hand, Lecuyer et al. show a down-regulation of placental VEGF-R1 in ethanol-exposed mice during the second trimester equivalent[209]. Oxidative stress may explain these findings[213,298]. Conversely, the PLGF is a member of the VEGF family and a key factor in angiogenesis and vasculogenesis (especially during embryogenesis) that is highly regulated depending on the developmental stage[212]. In this work, we found no differences in PLGF levels at GD19 in the different groups after continuous PAE. Upregulation of placental PLGF levels in response to PAE in the early stages of pregnancy may promote placental permeability[212], with progressive decrease[209] in comparison to the controls in later stages of pregnancy (when vasculogenesis is completed), as seen in this work. Changes in VEGF-VEGF-R levels and/or in the VEGF-R1/PLGF ratio lead to important imbalances in angiogenesis[299] (as demonstrated in other pathologies such as preeclampsia[299]), responsible for placental disorders and FGR. In addition, PAE-related oxidative stress may also contribute to angiogenesis deregulation and FGR.

EGCG is a natural antioxidant, as has been shown in studies assessing disorders related to oxidative stress[249]. Thus, EGCG may be a potential therapeutic tool for FASD. The results in our study indicate that EGCG ameliorates PAE-related FGR and attenuates ethanol-related changes in VEGF-A and VEGF-R1 expression, partially restoring the imbalance produced by PAE. Overall, EGCG exerts positive effects on the placenta and fetal growth in all ethanol exposed groups, more evident in the binge drinking group when VEGF-A, VEGF-R, PLGF, and fetal growth were analyzed. In this context, EGCG may be a therapeutic option to maintain adequate vascularization and promote correct angiogenesis[300] for adequate fetal growth.

PAE boosts the production of ROS and dysregulation of antioxidant systems, being one of the leading causes of FASD physiopathology[301]. The nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor involved in the expression of antioxidant proteins related to oxidative stress response[302]. Under oxidative stress conditions, Nrf2 is released from its inhibitor Keap-1 and translocated from the cytoplasm to the nucleus were it triggers the expression of genes encoding antioxidant proteins and detoxifying enzymes as catalase, superoxide dismutase, and the glutathione peroxidase families[303]. Hence, we used Nrf2 as a biomarker for oxidative stress in our experimental model. The intake of high doses of ethanol during fetal development increases Nrf2 antioxidant response, being the DG of the hippocampus the target for oxidative stress damage. However, no differences are observed in Nrf2 expression with the Mediterranean-like drinking pattern. Many studies have been carried out on the protective response of Nrf2 towards oxidative in adults, but are limited in fetal life. A previous study shows increased Nrf2 levels in the brains of mice embryos prenatally exposed to ethanol[303], similar to those determined in our binge-like drinking pattern group. Additionally, EGCG upregulates Nrf2 expression after different oxidative stress insults[302,304]. However, to date no studies have been performed on the prenatal effects of EGCG on Nrf2 after PAE. Surprisingly, in our study, presence of EGCG not only does not increase Nrf2 but it reduces it to physiological levels. Nrf2 activation is not triggered when EGCG and ethanol are given simultaneously during pregnancy and, probably because EGCG mainly prevents ethanol-induced damage through other molecular routes such as the induction of phase II detoxifying enzymes and scavenging ROS[283].

Ethanol-induced neurotoxicity is mainly attributed to oxidative stress and generation of ROS[229]. Throughout pregnancy, the CNS has vulnerable periods sensitive to alcohol damage that affect the developmental processes depending on the timing, dose, and pattern of exposure. Data presented in this thesis with the PAE mouse model reflect the loss of mature neurons (measured by the Neuronal Nuclei (NeuN) biomarker) in the DG and cerebellum in fetuses at GD19 for both human-like drinking patterns. Loss of mature neurons in the DG after alcohol exposure has been analyzed in previous studies with contradictory results. After PAE during the second trimester equivalent in a binge model in rats, the number of mature neurons were similar to the controls[116]; however, similar results to those presented in this thesis were found in adult rats prenatally exposed to ethanol during the first trimester equivalent[89]. Thus, the timing of exposure during fetal development may be a key factor for neuronal maturation processes. According to our results, alcohol-related reduction of mature neurons already occurs during fetal development. Further research is necessary to evaluate neurological and behavioral alcohol-related disorders secondary to neuronal loss in the DG and cerebellum during

neurodevelopment. In the same line, neuronal precursor cells and immature neurons express doublecortin (DCX) in embryonic and adult brain. Our results show increases of DCX+ neurons, particularly in the DG, in Mediterranean and binge-like drinking patterns. Similar findings were reported for different hippocampal regions in a PAE murine model during the second trimester equivalent[116]. In contrast, lower DCX quantification in the DG has been reported in comparison to the controls in adult mice prenatally exposed to ethanol[114]. Increased signaling in immature neurons during fetal life may indicate a delay in maturation processes produced by PAE-related oxidative stress damage on organogenesis[305,306]. As previously discussed, oxidative stress is one of the main molecular pathways of ethanol neurotoxicity in the developing organism; thus, EGCG may be effective for treating ethanol-related effects. EGCG therapy in fetal neurogenesis results in an improvement in maturation and differentiation processes. NeuN and DCX expression in the treated groups is comparable to that in controls, which indicates that EGCG may exert a beneficial effect on fetal neurogenesis. Studies with natural antioxidants in fetal life are scarce. Similar effects were shown on hippocampal neurogenesis in an adult mice neuroinflammation model, where EGCG treatment appears to be beneficial[307]. Consistently, a study in ethanol exposed adult mice demonstrated the compensatory effect of EGCG therapy on the affected immature neurons[307]. EGCG also seems to promote proliferation and differentiation, as evidenced by increased Ki67 and neuron-specific enolase expression[308,309]. These findings support EGCG as a potential therapeutic molecule to prevent delays in neurogenesis processes due to ethanol exposure in fetal life.

Differentiation is another important neurodevelopmental process disturbed by ethanol. GFAP is a glial cell to astrocyte differentiation biomarker used to evaluate disorders in this process. As reported in previous studies[310], our results show reduced astrocyte differentiation following PAE, reaching statistical significance in fetuses exposed to the human-like binge drinking pattern. Ethanol stimulates the early transformation of radial glia into astrocytes and their migration to ectopic areas[311]; thus, in more advanced stages of neurodevelopment, astrocyte differentiation is reduced. EGCG therapy elicited GFAP recovery to levels closer to physiological expression. Although there are no previous studies on the effect of EGCG on astrocyte differentiation in FASD-like animal models, results from other studies on neurodegenerative diseases show a neuroprotective effect of EGCG on astrocyte differentiation processes[312]. In vitro models show EGCG neuronal differentiation involvement through the protein kinase C pathway[313], inhibition of the glycogen synthase kinase-3 (GSK-3) pathway[314], or modulation of S100B[315].

The BDNF is involved in cell survival, development, and function of the CNS, representing one of the main biomarkers of neuronal plasticity during early development[316]. Disorders of neuronal plasticity may explain many of the neurobehavioral disorders in FASD individuals, such as learning and memory deficits[317]. EGCG has been selected as a potential pharmacological tool against FASD due to its ability to interact and inhibit neuronal plasticity inhibitors such as Dyrk1A[171] and potentiate NGF-induced neurite outgrowth[318,319]. Our study shows no statistically significant differences of BDNF levels after PAE or EGCG administration. On the contrary, Feng et al. reported a decrease of BDNF in rats prenatally exposed to a binge drinking pattern between GD5 and GD20, but no differences with lower alcohol doses[153]. Similarly, Haun et al. showed down-regulation of BDNF expression after the intake of large doses of alcohol (four-day with not-continuous ethanol administration) in an adult mouse model, and significant ethanol consumption decrease in the same established model of alcohol dependence after BDNF administration[165]. Our experimental design ensures continuous alcohol exposure, which leads to habituation to ethanol and therefore no change in BDNF expression. Further research to test the effects of EGCG therapy on BDNF expression in FASD-like models with not continuous ethanol administration are necessary.

To the best of our knowledge, the study “Epigallocatechin Gallate Ameliorates the Effects of Prenatal Alcohol Exposure in a Fetal Alcohol Spectrum Disorder-like Mouse Model”, submitted for publication as part of this thesis, is the first preclinical study that shows the potential pharmacological role of EGCG to mitigate a set of effects produced by prenatal alcohol exposure in a FASD-like mouse model. In the study, we show that EGCG treatment has beneficial effects on a wide range of FASD manifestations, such as placental development, fetal growth, oxidative stress, and neurodevelopmental processes. In addition, we compare the effects produced by the two human-like drinking patterns (Mediterranean and binge) on our mouse model. In general, any amount of alcohol produces alcohol-related effects in the offspring, but the most relevant effects are generally produced with higher doses of alcohol. Conversely, fetal growth is probably preserved with the human-like Mediterranean drinking pattern because lower doses of ethanol do not reach the threshold necessary to produce a FASD phenotype.

One of the limitations of our model is the experimental design based on continuous ethanol exposure throughout the pregnancy. This may have led to alcohol exposure adaptation in some critical processes related to FASD pathophysiology; however, we consider continuous ethanol exposure similar to alcohol abuse in humans.

Additionally, the experimental design did not allow assessing the effects of PAE that contribute to FASD in the third trimester equivalent.

Overall, the results presented here demonstrate the consequences of PAE considering two patterns of alcohol consumption, and the potential pharmacological role of EGCG during fetal development on a wide range of FASD manifestations (placental development, fetal growth, angiogenesis, oxidative stress, and neurodevelopmental processes). Further studies to evaluate the long-term effects of EGCG therapy on FASD phenotype are necessary.

FASD causes a variety of health care-related costs, imposing an enormous economic burden to society[320]. Thus, any novel FASD therapy should deserve special interest. To ensure successful outcomes it is essential to know the safety profile and pharmacokinetic parameters of EGCG[321] in humans. Important bioavailability differences have been reported for this molecule due to the heterogeneity of the human populations analysed[268,322]. The stability of EGCG also differs depending on the delivery strategy[270,323,324]. Additionally, absorption and stability of EGCG will be influenced when combined with other food products that determine the environment before EGCG absorption and modulate its biological response. Unfortunately, the interactions of specific nutrients with metabolic processes related to EGCG bioavailability remain unclear[272,325]. In order to determine the most appropriate conditions for EGCG intake we conducted a randomized, crossover study with healthy volunteers under three intake conditions: after overnight fasting, in combination with a Mediterranean diet breakfast, or as a dietary supplement.

Results of the second article of this thesis (Bioavailability of Epigallocatechin Gallate Administered with Different Nutritional Strategies in Healthy Volunteers) show differences in EGCG bioavailability depending on its ingestion under fasting conditions or accompanied with food. The highest bioavailability is obtained when Teavigo® (94% EGCG, 150 mg of green tea extract) is taken under fasting condition. Conversely, the lowest interindividual variability and higher stability in plasma concentrations are seen in individuals who are given FontUp® (sachets containing 94% EGCG, 266 mg of concentrated green tea extract plus fats, carbohydrates, proteins, vitamins, and minerals).

The highest peak concentrations (C_{max}) and AUC_{0-360} in both genders are obtained with a single 250 mg dose of EGCG (Teavigo®) administered under fasting conditions. The Mediterranean diet breakfast reduces EGCG bioavailability (in over 50% in males and 30% in females (AUCs)). Hence, green tea extract should be ingested alone after overnight fasting to optimize its gastrointestinal absorption. These results are consistent with previous studies showing that administration of EGCG alone elicits an

attenuated response from the stomach and pancreas, minimizing digestion[263,265]. Gastrointestinal processes are partially activated when EGCG is taken in the absence of other foods. EGCG remains stable in acidic environments (pH < 3) because its oxidation is reduced under this condition[326]. Moreover, the lack of EGCG neutralization by bile salts optimizes EGCG absorption by the enterocytes in the small intestine. Additionally, in the absence of activation of the digestion mechanisms, there is reduction of the gut microbiota responsible for catabolizing these antioxidant molecules in the large intestine. Accordingly, a standard Mediterranean breakfast (milk, cocoa, cereals, and toasts with olive oil) plus FontUp® reduces EGCG bioavailability. Significant decreases in EGCG bioavailability were reported in other studies when Teavigo® was taken with semi-skimmed milk[272,323,327]. Furthermore, Teavigo® in combination with breakfast, and FontUp® delayed the time (T_{max}) to peak plasma concentration (C_{max}). Simultaneous administration of EGCG with other foods delays the rate of gastric emptying (increased T_{max})[328]. On the other hand, EGCG molecules remain for longer periods in the more basic environment of the small intestine, which increases its degradation. Thus, the absorption of EGCG in presence of other nutrients is reduced/delayed as compared to EGCG alone. EGCG passes directly to the small intestine where it is absorbed.

We addressed the role of specific nutrients on EGCG bioavailability when taken simultaneously. We found interesting results when the food supplement FontUp® was compared against Teavigo® in combination with breakfast. As EGCG is unstable in environments with high temperature[329] and basic pH[330], our experiments were performed using fresh fluids at 4 ˚C to reduce ECGC degradation, water in the case of Teavigo® alone, and semi-skimmed milk for the other two series (FontUp® and Teavigo® plus breakfast). Ascorbic acid (vitamin C) has been used as preservative to increase EGCG bioavailability by preventing its oxidation at an acidic pH[265,271,331]. Vitamin C maintains the polarity of the eight hydroxyl groups and the structural stability of the molecule. The ratio used in FontUp® (12 mg of ascorbic acid per 250 mg of pure EGCG) provided the best stability parameters, measured by $T_{1/2}$, after gastrointestinal absorption compared to Teavigo® under fasting conditions and Teavigo® in combination with breakfast. EGCG blood levels, measured by C_{av}, were five-fold lower with FontUp® compared to Teavigo® alone, and 2.5-fold lower than Teavigo® plus breakfast. Thus, presence of vitamin C in FontUp® did not improve EGCG absorption as did the presence of either sucrose[271]. Moreover, omega-3 polyunsaturated fatty acids from fish oil have also been shown to improve intestinal absorption of EGCG[270,332,333]. However, their presence in FontUp® does not improve EGCG bioavailability compared to Teavigo® under fasting conditions or after a standard breakfast. New delivery modes have been proposed aiming at improving oral EGCG bioavailability to expand its use in a lipophilic environment.

Esterification of the water-soluble EGCG with stearic acid, eicosapentaenoic acid, and docosahexaenoic acid, or replacing the hydroxyls of EGCG with acetyl groups, have led to significant improvements in stability, bioavailability, and antioxidant or antiproliferative bioactivities[274,276]. Their potential applications in future clinical studies should be analyzed in more detail.

The main limitation of the previously discussed study is the low EGCG bioavailability estimated in humans (0.1%–0.3%)[260,261] as the primary cause of interindividual variability. In order to reduce the impact of interindividual variations, we designed a crossover study in which each volunteer was its own control. EGCG has been tested for the treatment of several pathologies in a range of doses from 150 to 400 mg per day, being oral administration the most widely used delivery method in animal models and human clinical trials[325,334,335]. In our study, we determined the optimal conditions for EGCG administration based on the objectives of the research. High concentrations of Teavigo® must be used under fasting conditions when high experimental concentrations of EGCG are needed. On the other hand, Teavigo® taken with a Mediterranean breakfast or FontUp® are useful for studies in which reduced interindividual variability is required. Further research on new modes of delivery to improve EGCG oral bioavailability may optimize its disposition.

Overall, this thesis adds new insights to previous evidence regarding FASD therapy. Alcohol-related health problems are a major hot topic because of the social and economic impact. Recent prevalence studies reveal worrying data on prenatal alcohol exposure, with consumption during pregnancy of up to 65% for any amount of alcohol in some populations[16]. The results presented in this PhD thesis show that prenatal alcohol use in mouse models simulating the different human-like patterns may produce detrimental effects in the offspring. Alcohol-related effects include alterations in placental development with underlying fetal growth restriction, as well as neurodevelopmental disorders that can lead to cognitive disabilities and behavioral disorders in childhood and adult life. Moreover, we show that prenatal use of EGCG may ameliorate FASD. These results encourage further research to find the molecular pathways involved in EGCG protective effects, as well as postnatal studies to evaluate its effects on FASD-related behavioral disorders. Finally, understanding the optimal pharmacokinetic conditions for EGCG treatment will allow its translation to pregnant women as a safe alternative[336,337] aiming to ameliorate FASD-related health, as well as the social and economic burden.

CONCLUSIONS

6. CONCLUSIONS

1. Any type of alcohol drinking pattern (Mediterranean or binge) produces alcohol-related effects in the offspring.

2. There is an inverse relationship between fetal growth and prenatal alcohol exposure. Fetal growth is preserved with the human-like Mediterranean drinking pattern because the ingested alcohol doses do not reach a threshold that causes fetal growth restriction. However, any alcohol drinking pattern leads to imbalances of placental angiogenic factors.

3. Any type of alcohol drinking pattern produces losses of mature neurons, delay in neuronal maturation processes, and disorders in astrocyte differentiation. Continuous prenatal alcohol exposure may lead to habituation in some neuronal processes such as neuronal plasticity.

4. EGCG may potentially be used for the treatment of fetal alcohol spectrum disorders as it seems to improve placental development and fetal growth, angiogenesis, oxidative stress, and neurodevelopmental processes.

5. The highest bioavailability for EGCG is reached in both genders with oral administration under fasting conditions of green tea extracts (Teavigo®) with a high percentage of this compound.

6. Teavigo® taken with a Mediterranean diet breakfast or the food supplement FontUp® reduces interindividual variability.

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7. REFERENCES

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APPENDIX I

UNIVERSITAT DE BARCELONA

C.E.E.A
(Comitè Ètic d'Experimentació Animal)

Centres Científics i Tecnològics
Edifici Florensa, C/Adolf Florensa nº8, 1ª planta, despatx F14
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ceea@ccit.ub.edu

COMITÈ ÈTIC D'EXPERIMENTACIÓ ANIMAL (CEEA)

Formulari d'acceptació de procediments

DADES PROCEDIMENT

Títol: **Estudio comparativo del trastorno del espectro de alcohol fetal en ratones según dos patrones de consumo humano (consumo moderado y consumo agudo) y de la restricción de crecimiento fetal y los efectos de la administración de –(-)epigallocatechin-3-gallate.**

Investigador Responsable: **LAURA ALMEIDA**

Un cop examinada la documentació presentada, en compliment del Decret 214/97 de la Generalitat de Catalunya, el CEEA de la UB ha resolt **ACCEPTAR** el procediment sol·licitat.

Signat pels membres del CEEA que han pres l'acord

Barcelona, 12 de febrer del 2015

NOTA: El CEEA delega en el/la responsable en benestar animal de la Unitat d'Experimentació Animal on s'allotjaran els animals, el seguiment de la realització del procediment d'acord amb el que està establert a la memòria aprovada per aquest comitè.

Generalitat de Catalunya
Departament d'Agricultura,
Ramaderia, Pesca i Alimentació
Serveis Territorials a Barcelona
Secció de Biodiversitat i Activitats Cinegètiques

UNIVERSITAT DE BARCELONA
CEEA-UB (Comitè Ètic d'Experimentació Animal)
A/A Álvaro Gimeno Sandig
Edifici Florensa, c/ Adolf Florensa nº 8, 1ª planta, despatx F14
08028 Barcelona

Assumpte : Comunicació prèvia de procediments d'experimentació animal
(expedient B-NPP-372/15)

Senyor,

Hem rebut la comunicació prèvia del procediment d'experimentació:

Títol: " Estudio comparativo del trastorno del espectro de alcohol fetal en ratones C57BL/6 según dos patrones de consumo humano (consumo moderado y consumo agudo) y los efectos de la administración de epigallocatechin-3-gallate a corto plazo "

Investigador/a responsable: Laura Almeida Toledano

Aquest procediment té assignat el número d'ordre DARP: **8744.**

D'acord amb l'article 32 del Decret 214/1997 de 30 de juliol sobre protecció dels animals utilitzats per a experimentació i altres finalitats científiques, aquest procediment té una validesa fins el **17 de desembre de 2017**, sempre i quan no existeixi cap modificació del procediment.

Atentament,

El Cap de la Secció de Biodiversitat
i Activitats Cinegètiques

Josep Maria López Martín

Barcelona, 20 de gener de 2016
ilp

REGISTRE
C.E.E.A.
Comitè Ètic d'Experimentació Animal
Data: 2 3 GEN. 2016
Entrada: 23/16
Sortida: —

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Fundació Sant Joan de Déu

CEIC Fundació Sant Joan de Déu

Dr. Pau Ferrer Salvans
Secretario del CEIC Fundació Sant Joan de Déu

CERTIFICA

1º. Que el CEIC Fundació Sant Joan de Déu en su reunión del día 22/02/2018, ha evaluado la propuesta del promotor referida al estudio:
Título: "Estudio de biodisponibilidad de epigalocatequina-3-galato (EGCG) (FontUp®)"
Código Interno: PIC-11-18
IP: Doctora Maria Dolores Gómez Roig

Considera que:

- El proyecto se plantea siguiendo los requisitos de la Ley 14/2007, de 3 de julio, de Investigación Biomédica y su realización es pertinente.
- Se cumplen los requisitos necesarios de idoneidad del protocolo en relación con los objetivos del estudio y están justificados los riesgos y molestias previsibles para el sujeto.
- Son adecuados tanto el procedimiento para obtener el consentimiento informado como la compensación prevista para los sujetos por daños que pudieran derivarse de su participación en el estudio.
- El alcance de las compensaciones económicas previstas no interfiere con el respeto a los postulados éticos.
- La capacidad de los Investigadores y los medios disponibles son apropiados para llevar a cabo el estudio.

2º. Por lo que este CEIC emite un **DICTAMEN FAVORABLE**.

3º. Este CEIC acepta que dicho estudio sea realizado en los siguientes CEIC/Centros por los Investigadores:

- **HOSPITAL SANT JOAN DE DEU. Maria Dolores Gómez Roig.**

Lo que firmo en Esplugues de Llobregat, a 6 de marzo de 2018

Fdo:

Fundació Sant Joan de Déu
Comitè Ètic d'Investigació Clínica

Dr. Pau Ferrer Salvans
Secretario del CEIC Fundació Sant Joan de Déu

APPENDIX II

frontiers in Pediatrics

REVIEW
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Murine Models for the Study of Fetal Alcohol Spectrum Disorders: An Overview

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Prenatal alcohol exposure is associated to different physical, behavioral, cognitive, and neurological impairments collectively known as fetal alcohol spectrum disorder. The underlying mechanisms of ethanol toxicity are not completely understood. Experimental studies during human pregnancy to identify new diagnostic biomarkers are difficult to carry out beyond genetic or epigenetic analyses in biological matrices. Therefore, animal models are a useful tool to study the teratogenic effects of alcohol on the central nervous system and analyze the benefits of promising therapies. Animal models of alcohol spectrum disorder allow the analysis of key variables such as amount, timing and frequency of ethanol consumption to describe the harmful effects of prenatal alcohol exposure. In this review, we aim to synthetize neurodevelopmental disabilities in rodent fetal alcohol spectrum disorder phenotypes, considering facial dysmorphology and fetal growth restriction. We examine the different neurodevelopmental stages based on the most consistently implicated epigenetic mechanisms, cell types and molecular pathways, and assess the advantages and disadvantages of murine models in the study of fetal alcohol spectrum disorder, the different routes of alcohol administration, and alcohol consumption patterns applied to rodents. Finally, we analyze a wide range of phenotypic features to identify fetal alcohol spectrum disorder phenotypes in murine models, exploring facial dysmorphology, neurodevelopmental deficits, and growth restriction, as well as the methodologies used to evaluate behavioral and anatomical alterations produced by prenatal alcohol exposure in rodents.

Keywords: prenatal alcohol exposure, fetal alcohol spectrum disorders, fetal alcohol syndrome, alcohol consumption patterns, facial dysmorphology, neurodevelopmental disorders, fetal growth restriction, models of fetal alcohol spectrum disorders

INTRODUCTION

Alcohol is a known teratogen. Its frequent use during pregnancy impacts the normal development of human fetuses promoting severe developmental alterations and generating a wide range of physical, behavioral, cognitive, and neurological impairments. In 1968, Lemoine et al. established an association between prenatal alcohol exposure (PAE) with certain neurodevelopmental disabilities (1). However, it was not until 1973 when Jones and Smith provided the initial characterization of fetal alcohol syndrome (FAS) (2), defined as growth restriction, facial dysmorphologies (wide-spaced eyes, mid-facial hypoplasia, and a smooth philtrum), and central nervous system (CNS) disorders, resulting in motor, cognitive and behavioral disorders (3). Subsequent observational studies identified and characterized the umbrella term fetal alcohol spectrum disorder (FASD) (4) that includes: FAS (the most deleterious manifestation of FASD), partial FAS (pFAS) (an intermediate phenotype defined by the absence of some FAS characteristics), alcohol-related birth defects (ARBD) (certain physical impairments are exhibited), and alcohol-related neurological disorders (ARND) (behavioral and learning neuropsychological alterations, usually without facial dysmorphology) (5).

Thus, behavioral deficits in FASD subjects associate with structural changes in brain organogenesis: the *Corpus callosum* may lose its structure (agenesis) and generate cognitive deficits linked to attention, executive and psychosocial functions, language, and reading comprehension (6); cerebellum and anterior part of the vermis may suffer hypoplasia and affect motor skills and learning capacity (7). Moreover, proven asymmetry of the hippocampus in FAS children may also affect their memory (8). The degree of structural abnormalities in the brain correlates with the severity of FAS-like facial features, and this in turn, with more serious behavioral problems (9).

According to the World Health Organization, PAE is the main preventable cause of intellectual disability in the western world (10–12). A recent meta-analysis estimated global prevalence of alcohol use during pregnancy to be 9.8% (13). Therefore, PAE-related disorders may lead to major problems for the social environment as well as economic setbacks for the public health system.

Animal models play a key role in the study of FASD by allowing the development of novel diagnostic and therapeutic tools. Researchers have used a great variety of organisms to mimic the physical and behavioral characteristics found in PAE and FASD phenotypes. Inbred strains of rodents are genetically homogenous populations that facilitate result reproducibility and interpretation in studies designed to evaluate the impact of environmental insults such as ethanol. Moreover, the alcohol intake pattern can be more precisely defined (timing and dose), allowing the identification of time-sensitive windows and thresholds of harmful doses during pregnancy. Rodents have been widely used in FASD research to assess the way PAE-related impairments affect metabolic pathways, molecular biology, cell signaling, synaptic plasticity, and cognition during fetal development, promoting the study of variables affected by alcohol exposure at neuroanatomical, neurochemical and behavioral levels (14).

In this review, we focus on rodent FAS-like phenotype neurodevelopmental disabilities, taking into account facial dysmorphology and fetal growth restriction. We examine every stage of brain development, considering changes caused by PAE in different neural cell lineages, molecular pathways and oxidative stress epigenetic variations. We also review the experimental methodologies used to generate rodent FASD-like phenotypes, including advantages and disadvantages of the different routes by which alcohol has been administered. Finally, we revise anatomical and behavioral alterations, as well as the methodologies used to assess these features in murine models.

FASD-LIKE ANIMAL MODELS

FASD studies in humans have common limitations due to the complexity in correctly measuring certain variables such as maternal diet or health, or the volume and timing of ethanol exposure during pregnancy. These difficulties may be resolved by using animal models, simple, effective, and reliable tools for alcohol research. These models are useful for understanding the molecular mechanisms underlying alcohol teratogenicity and for monitoring cognitive and behavioral changes. Animal models also allow assessing different therapeutic approaches in preclinical studies, for initial screening of the compounds and strategies for future human clinical studies.

The invertebrate *Caenorhabditis elegans* is a simple model for development and is commonly chosen to study the effects of ethanol on molecular pathways. However, the embryos develop outside the body, exact ethanol concentrations administered cannot be finely controlled (15), and the way alcohol is metabolized differs substantially from that in humans (15). The zebrafish (*Danio rerio*) has several physiological and genetic similarities with humans (16), which makes it a suitable alternative as model of vertebrate. Regarding the effects of ethanol, there are further advantages: substantial knowledge of all stages of development, short developmental period, and produce large amounts of offspring (17). Zebrafish eggs and embryos are transparent (just like in nematodes) making embryonic development easy to follow, facilitating exposure to alcohol of the embryos during different and precise developmental periods, and easy determination of physical malformations and simple behaviors (16, 18). By contrast, the chorion of the egg acts as a barrier and large volumes of ethanol are necessary to ensure its penetration (17).

Mammals offer significant advantages in the study of brain structures or complex behaviors (19). Although primates could be the gold standard, there are some disadvantages, mainly the long duration of the studies and ethical limitations (19). Rodents are the most employed mammals for FASD research because they are easy to handle, have a short gestational period, and produce large numbers of offspring. Rats offer the advantages of being larger and with a more sophisticated behavior in comparison to mice. Regardless, mice (particularly the C57BL/6 strain) are the most commonly used mammal

due to their ease of care, availability of transgenic and disease models, short lifespan, and basic physiology and genetics similar to that of humans. Teratogenic effects of alcohol exposure in mice have been reported, including craniofacial malformations, altered neurogenesis processes, and soft-tissue and skeletal abnormalities (20, 21). The main disadvantage in using rodents for FASD research is that the third trimester equivalent to human development in rodents occurs after birth. Thus, there are differences in the processes of absorption, distribution, metabolism and elimination in rodents in comparison to the human utero, with no influence of the placental barrier. Interestingly, C57BL/6J is the strain with the highest preference for alcohol (22).

In following sections, we discuss details that need to be considered when a murine model is chosen for a FASD study.

Alcohol Exposure Patterns

Drinking patterns are characterized by the amount and frequency of ethanol taken. This is measured by blood alcohol concentration (BAC) and expressed as weight of alcohol per unit of volume of blood.

Kelly et al. showed that binge-like alcohol exposure is more harmful than non-binge exposure in rat brain development after exposure to the same dose of ethanol. The authors administered doses of 6.6 g/kg/day of ethanol to neonatal rats using artificial rearing, following one of two possible patterns. A continuous pattern (24 h per day) for several days, which resulted in an average BAC peak of 79–97 mg/dL or an acute exposure pattern (8 h per day) for the same period of time, resulting in an average BAC peak of 56–415 mg/dL. Lower brain growth was observed in the acute exposure group in comparison to the continuous pattern (23). Other findings support the hypothesis that lower daily doses of ethanol following a binge-like pattern leads to lower brain weight and cell loss in different brain areas than higher non-binge doses. Three groups of ethanol-exposed rat pups were compared. One group was exposed to 4.5 g/kg/day in a condensed pattern (4 h per day), the second group was exposed to the same dose although administered in a less condensed pattern (8 h per day), and the third group was exposed to a higher dose of alcohol (i.e., 6.6 g/kg/day) administered in a continuous pattern (24 h per day). The resulting average BACs peaks were 361, 190, and 39 mg/dL, respectively. The authors found that pups exposed to 4.5 g/kg/day over 4 h had the lowest brain weight, followed by the second group. The animals that ingested highest doses of ethanol throughout the 24 h had the highest brain weights (24). These results demonstrate that ethanol intake under a binge-like pattern is more harmful than higher doses taken for longer periods of time due to higher BAC peaks in shorter periods of time.

Control Group

Several studies have assessed the influence of nutritional intake on the teratogenic effects of alcohol (25, 26). Alcohol can replace other nutrients because of its caloric content and may interfere with the absorption of other nutrients due to its inflammatory effects on the stomach (27).

Pair-fed control has been used in some FASD-like animal model experiments since it acts as a calorie-matched control group. A carbohydrate substance (e.g., maltose dextrin or sucrose) is usually employed to replace ethanol-derived calories in the diet (28). A pair-fed group may also allow monitoring a stress condition. On the other hand, the pair-fed group is considered as an imperfect control group, since the pattern of food consumption in this group is different from a physiological intake. Individuals in pair-fed controls consume the assigned food as soon as it is available, creating additional stress associated to food restriction. In addition, in the pair-fed group it is not possible to match the effect of alcohol on the absorption of other nutrients because of its inflammatory effects. Thus, some researchers have suggested the use of a basal control group known as non-handle, *ad libitum*, or *sham*, in which the intake of nutrients resembles the physiological one. This is useful to avoid biases caused by ethanol interference in nutrient absorption (29). Consequently, the use of a pair-fed group and an *ad libitum* control group should be considered as an alternative when designing a FASD murine model study.

Route of Administration and Dosage Forms

Several modes of ethanol administration methods have been described, particularly in rodent gestation. Ethanol delivery methods directly affect variables such as the alcohol exposure pattern, exact amount of alcohol taken, and generated stress. All these variables must be taken into account during experimental design. Voluntary ethanol feeding and intragastric gavage are the most physiological administration methods. Voluntary ethanol feeding (30, 31) is a safe technique when low stable BAC levels want to be reached. Conversely, intragastric gavage (29) offers a more accurate control of doses and timing, and reaches higher BACs. Inhalation (32) or injection (33) offer some advantages compared to voluntary ethanol drinking and intragastric gavage due to their time efficiency. Artificial rearing is a useful method when the aim of the study is alcohol administration in a third trimester equivalent model (29, 34, 35). Briefly, the choice of method must consider the purpose of the experiment and the researcher's experience. **Table 1** [based on a previous review (45)] summarizes the characteristics of the main routes of alcohol administration in rodents and dosage forms, focusing mainly on mice.

Blood Alcohol Concentration

BAC depends on several factors such as dosage, pattern of exposure, metabolic rate, food consumption, tolerance and genetics (46, 47). As mentioned above, BAC peaks are higher when ethanol is administered in a binge-like pattern, even with low doses of alcohol (24). There are several types of methods for measuring BAC: headspace gas chromatography (HS-GC), headspace solid-phase microextraction (HS-SPME), capillary gas chromatography, or enzymatic ADH immunoassays (48). Immunoassays are not as accurate as mass spectrometry and are susceptible to bias by overestimating alcohol concentration due to non-specific interferences. On the other hand, immunoassays are sufficiently accurate, easy to use in any laboratory, and require a small amount of sample ($\sim$100 μL). Immunoassays

TABLE 1 | Characteristics of the different routes of ethanol administration in mice.

Administration route	Characteristics	Reached BAC	Advantages	Disadvantages
Voluntary ethanol feeding	Oral, self-administration. Pre-gestational alcohol consumption is usually introduced (36). Sometimes, ethanol is added to flavored liquid nutritional formulas (Liquid-diet or Sustacal) to allow easy self-administration (37, 38). 10–20% (vol/vol) ethanol solution (36). Possibility of isovolumic and isocaloric pair-fed diet (e.g., maltose-dextrin) in controls (28). Drinking in the dark (DID) procedure mimics binge-like pattern (39).	50–100 mg/dL when ethanol intake is 1–2 g/Kg [10% (vol/vol) ethanol solution]	Prevent the stress caused by other invasive methods. Safe technique. Easy to carry out. Gradual BAC increase. Low, stable BAC levels. Used prenatally.	Lower ethanol BAC achieved compared to other administration routes. Not useful for binge drinking pattern. Difficult control of dose and timing. No proper control of dose in breastfeeding pups. Not recommended postnatally. Lower BAC achieved if saccharin or a sucrose-sweetened solution is added to the alcohol.
Intragastric gavage	Administration of ethanol into the stomach using a gavage needle. Administered volumes <2 mL/100 Kg body weight (40). Allowed alcohol concentration <31.5% (vol/vol) (40). Ethanol dose 2–6 g/Kg/day (28). Ethanol vehicle (water, saline solution, or nutritional formula) (28).	250–300 mg/dL (60 min) for administration doses of 3.8 g/Kg [21% (wt./vol) ethanol solution]	Useful for binge drinking pattern. Accurate control of dose and timing. Reliable high BAC. Useful for pre- and postnatal administration.	Inhibition of suckling behavior in neonates. Stressful procedure for animals. Invasive procedure.
Inhalation	Inhalation chamber filled with ethanol vapor (41). Sometimes, administration of pyrazole to obtain stable BACs (32, 42).	150–250 mg/dl when volatized ethanol (ethanol 95%) is delivered to the chamber at a rate of 10 l/min	Reliable high BAC. Not a stressful technique for animals. Time and labor efficient. Useful for pre- and postnatal administration. Higher BACs in neonates compared to mothers.	Does not mimic the routes of intake in humans. Special equipment required. Interindividual variations.
Intraperitoneal injection	Ethanol solution injection in intraperitoneal space (43). Single or multiple doses for several days during pregnancy.	350–400 mg/dL (60 min) for administration doses of 3.8 g/Kg [21% (wt./vol) ethanol solution]	Rapid increase in BAC. Time efficient. Useful for pre- and postnatal administration. Useful for binge drinking pattern.	Handling-induced stress. Different intake routes in humans. This administration route produces higher BAC in fetuses than other routes using the same PAE. Higher incidence of malformations when used during first trimester equivalent.
Artificial rearing	Intragastric gavage ethanol discharge in pups while being kept in a special setting to mimic maternal environment (29, 34, 44). Placement of gastrostomy catheters.	150 mg/Kg when ethanol solution of 2,5 g/Kg is administered or 420 mg/Kg when ethanol solution of 7,5 g/Kg is administered*	Accurate control of dose and timing. Useful for postnatal administration. Mimics human third trimester.	Invasive and expensive technique. Social factors removed due to isolation of pups.

**Data obtained from experiments with rats (no available data for mice). BAC, blood alcohol concentration; Vol, volume; Wt, weight.*

are currently the most commonly used method for determining BACs in peripheral blood.

In animal models, BAC is defined as the amount of ethanol per unit of blood (usually mg/dL), measured when ethanol concentration reaches the highest level in peripheral circulation (49). In rodents, peak concentration is detected between 30 and 150 min (50–100 min in mice and 50–150 min in rats) following administration. The timeline of BAC depends on the administration route, the dosage and the species (rate of ethanol metabolism is 550 and 300 mg/Kg/h in mice and rats, respectively) (50). Severe neurotoxicity is typically linked to binge-like episodes causing higher BACs (i.e., BAC over 300 mg/dl in rats). However, continuous alcohol exposure, reaching lower BAC levels (i.e., BAC below 40 mg/dl in rats) despite higher doses, induces more subtle brain injuries (23, 24).

DEVELOPMENTAL STAGES OF THE FETAL BRAIN

During the development of the CNS throughout pregnancy, there are vulnerable periods sensitive to environmental insults. PAE affects brain organogenesis differently depending on the dosage, timing, developmental stage (moment), and location of the cell

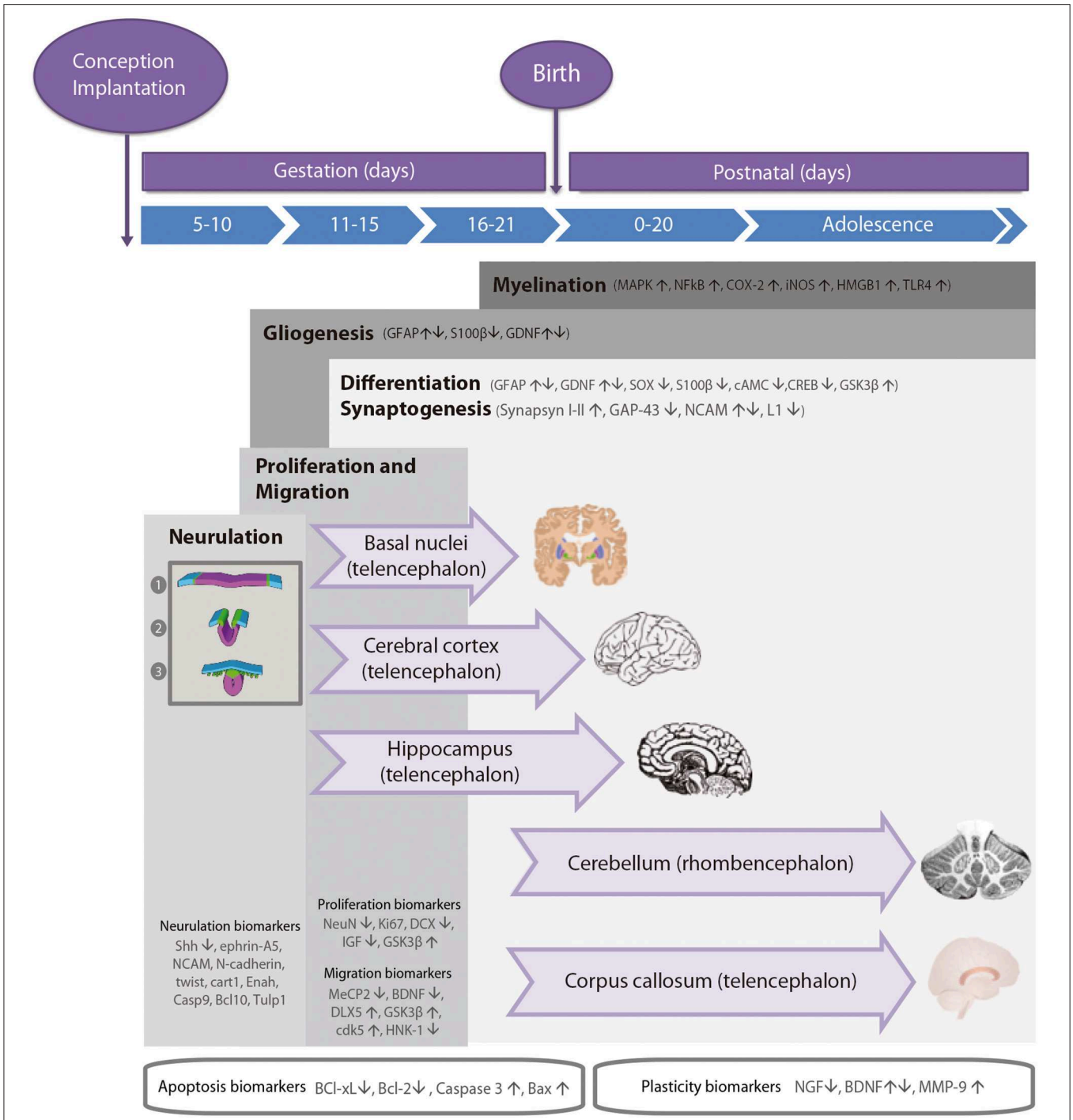

FIGURE 1 | Timeline of neurodevelopmental processes and brain neurogenesis in fetal rodents by areas sensitive to alcohol injury. Proteins in neurulation, proliferation, migration, differentiation, synaptogenesis, gliogenesis, and myelination neurodevelopmental processes. Changes in the levels of these biomarkers (up- or down-regulation) caused by prenatal alcohol exposure are represented by arrows. Stages of neurulation: (1) Neuroectodermal tissues differentiate from the ectoderm and thicken into the neural plate. The neural plate border separates the ectoderm from the neural plate. (2) The neural plate bends dorsally, with the two ends eventually joining at the neural plate borders, forming the neural crest. (3) The closure of the neural tube disconnects the neural crest from the epidermis. Neural crest cells differentiate to form the peripheral nervous system.

types involved in the biological stages (**Figure 1**). Key processes such as proliferation (51), migration (52), differentiation (53), synaptogenesis (54, 55), gliogenesis, myelination (56), and apoptosis (57, 58) are altered by PAE leading to congenital abnormalities and functional deficits in the CNS during fetal development (**Figure 2**) (74, 75).

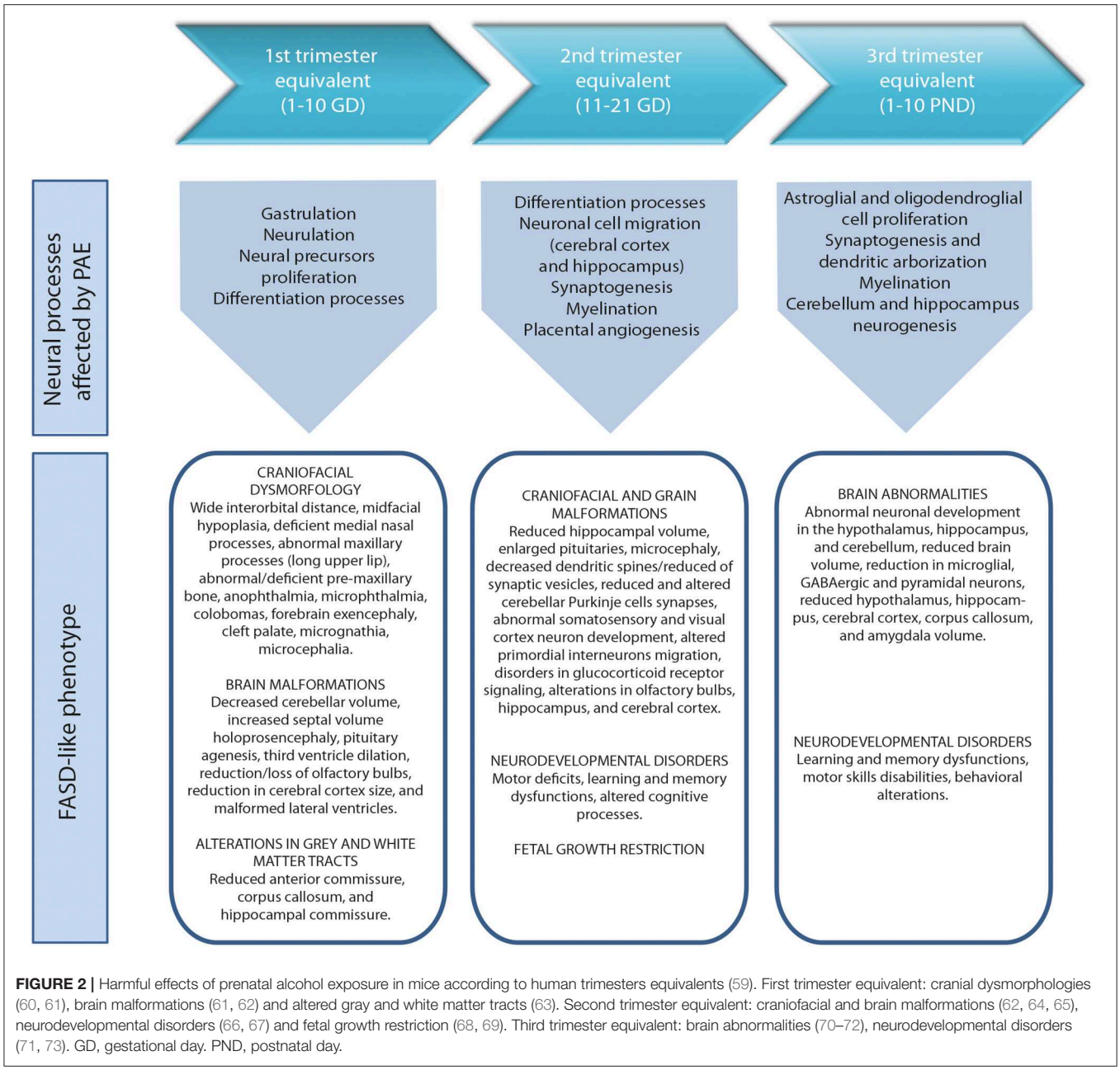

FIGURE 2 | Harmful effects of prenatal alcohol exposure in mice according to human trimesters equivalents (59). First trimester equivalent: cranial dysmorphologies (60, 61), brain malformations (61, 62) and altered gray and white matter tracts (63). Second trimester equivalent: craniofacial and brain malformations (62, 64, 65), neurodevelopmental disorders (66, 67) and fetal growth restriction (68, 69). Third trimester equivalent: brain abnormalities (70–72), neurodevelopmental disorders (71, 73). GD, gestational day. PND, postnatal day.

The anatomies of human and rodent brains show analogous structures and similar stages of development. However, they also exhibit some anatomical and functional differences. Human pregnancy consists of three pre-natal trimesters in which the brain rapidly grows between week 25 and 38. Several differentiation and proliferations processes occur in the third trimester of gestation, with maximum brain growth rate at birth and gradual decrease in early life (59, 76). Rat and mouse pregnancies are shorter than human pregnancies (rats: 21–23 days; mice: 20–22 days) and newborns undergo substantial brain development following birth (56, 57). The first trimester (59) in human pregnancy corresponds to gestational days (GDs) 1–10/11 in rat and mouse. The second trimester equivalent corresponds to GDs 11–21/22 (mice usually give birth on GD 21 and rats on GD 22), and the third trimester equivalent correlates to postnatal days (PNDs) 1–10. The ontogeny of specific behaviors can be used to draw inferences regarding the maturation of specific brain structures or neural circuits in rodents and humans. Despite the similarities between human and rodent brain development it is important to consider that rodents do not exactly mimic the developmental phases of human gestation (**Figure 1**).

Cellular precursors of the brain and the spinal cord develop through neurulation in early embryogenesis (**Figure 1**). The cellular fate of neurulation is the formation of the notochord,

which defines the primitive axis of the embryo and determines the vertebral system. The neural tube closure starts in the hindbrain area above the origin of the notochord, and continues anteriorly and posteriorly, making a caudal-to-rostral gradient in the developing brain. Neural tube formation finishes at gestational day (GD) 10–11 in rodents (77). Early in the second week of pregnancy (GD 7 in mouse, GD 9.5 in rats), neurogenesis and subsequent cell migration shape specific areas of the CNS in the forebrain, midbrain, and hindbrain, promoting distinct series of developmental processes (77). Therefore, the second critical developmental stage for PAE occurs between GD 5 and 11, implying alterations in organogenesis, neural tube formation and proliferation of neuronal precursors in areas adjacent to the neural tube. High levels of alcohol exposure during this stage not only cause major neural tube defects, but also lead to facial dysmorphologies similar to those observed in children affected with FAS.

The second critical developmental stage occurs between GD 11 and 21. During this period, most CNS areas are involved in distinct differentiation processes and several neuronal cell types emerge and migrate to specific areas of the brain (including the cerebral cortex and the hippocampus; **Figure 1**). The developmental phase of the different cell lineages varies according to its spatial location in separate brain areas. PAE particularly affects the neurulation, proliferation, and migration processes of the neocortex, cerebellum, hippocampus, and the basal ganglia. The last decisive developmental period occurs from GD 18 to postnatal day (PND) 9 and is characterized by the proliferation of astroglial and oligodendroglial cells, synaptogenesis, and dendritic arborization, which produce an increase in brain weight. At the same time, neurogenesis continues in the cerebellum and the dentate gyrus (DG) of the hippocampus. Alcohol exposure during third trimester induces severe neuronal loss, reactive gliosis, impaired myelination, as well as damage to the prefrontal cortex, hippocampal and cerebellar regions (64, 78, 79).

Proliferation

Neurogenesis is a highly regulated process whose timing and phases depend on the anterior-posterior gradient in the neuronal axis and the regions of the brain formed during organogenesis. Most cell proliferation processes take place throughout all the stages of neurodevelopment (80), although the most expansive phase occurs in the second half of pregnancy (mice: GD 10–21, rats: GD 11–22). This is a key developmental period due to ethanol toxicity vulnerability of neuronal precursors and brain structures (51), which may cause permanent alterations and profound behavioral deficits. For that reason, the consequences of PAE on proliferation and differentiation processes are assessed not only during fetal development but also later in life. As shown in **Figure 1**, several biomarkers help identify and evaluate neurogenesis and proliferation processes during neurodevelopment.

NeuN is expressed in nearly all post-mitotic neurons representing a reliable marker of mature neurons (81). This protein may also act as a biomarker of neuronal integrity, as it decreases in brain regions such as the hippocampus following ethanol exposure in rats (81–83). Ki67 has been thoroughly analyzed as a proliferation biomarker during neurogenesis in PAE studies.

Several authors have studied the effect of PAE on different regions of the hippocampus with different results. Some have found a reduced number of granular cells in the DG and pyramidal cells in specific regions of the rat hippocampus after GD 1–GD 20 plus PND 4–10 of ethanol exposure (24, 84), and during the third trimester equivalent (24, 83, 84), with no changes in the number of hippocampal neurons after GD 1–20 ethanol exposure (84, 85). Komada et al. showed a reduced proliferation rate (measured by Ki67) in mouse telencephalon after PAE on GD 6–18 (86). Conversely, West et al. showed an increase in the number of granular cells of the DG in rat hippocampus after ethanol exposure during the third trimester equivalent (87). Thus, early disturbances in proliferation after PAE may differ depending on the developmental period in which exposure to ethanol occurs.

Some of the effects of PAE on the hippocampus can be identified from birth, but others are more subtle and difficult to detect in the early stages. The consequences of PAE on hippocampal cell proliferation and survival in young adult animals are not always persistent (29, 35, 88). Interestingly, no changes in hippocampal cell proliferation (assessed by Ki67 and BrdU), but an increase in immature neurons of adult hippocampus in rats prenatally exposed to alcohol, have been described, probably due to a compensatory mechanism against PAE effects (29). Other authors have shown alterations in cell proliferation [measured by Ki67 (35) and BrdU (35, 88)] and increased neuronal maturation in the DG of the hippocampus in young adult rats prenatally exposed to ethanol. More recently, Gil-Mohapel et al. described significant decreases in adult hippocampal neurogenesis in aged rats after PAE (during first and second trimester equivalent), not previously seen in younger animals. These findings suggest a more conserved neurogenesis capacity in the early stages of life (89). Moreover, Delatour et al. analyzed Ki67 levels in pyramidal cells in adolescent mice exposed to ethanol at GD 13.5–16.5 and showed there were no changes when compared to controls (90). Once again, it seems that alterations in hippocampal neurogenesis vary according to the timing of ethanol exposure.

Coleman et al. examined the long-term effects on adult hippocampal neurogenesis after ethanol exposure in PND 7 in male and female mice. Increased Ki67 levels were found in the DG in males but not in females (70). This reveals gender differences regarding susceptibility to PAE.

PAE also affects the activity of enzymes involved in neurogenesis and proliferation, promoting hippocampal function behavioral disorders. Glycogen synthase kinase-3β (GSK3β) is highly expressed during brain development [from GD18 to PND10 in rats (91) and GD16 to PD18 in mice (92)] modulating different developmental processes such as neurogenesis, differentiation and neuronal survival. GSK3β activation sensitizes neurons to ethanol-induced injury, deregulating cell proliferation mechanisms (93). Increased levels of GSK3β post-PAE activates apoptosis in neural progenitor cells, decreasing neurogenesis and differentiation in immature

brains. Additionally, ethanol decreases the insulin-like growth factor (IGF) receptor signaling, affecting neural proliferation and decreasing the transcription of c-myc, c-fos, and c-jun51 in cell cultures (94).

Current evidence indicates that prenatal and neonatal alcohol exposure reduces the number of mature and immature neurons. Interestingly, this reduction is subtle when ethanol exposure is not continuous. Nonetheless, the brain region, developmental stage, and cell type are key factors when analyzing results of the biomarkers in proliferation processes.

Migration

Migration from the ventricular and germinal layers occurs radially in the medial/dorsal neocortex and tangentially in other regions of the forebrain (95). On GD 5, superficial layers are still not clearly defined (96). On GD 14.5 (mice) or GD17 (rats), the first cell lineages reach the area that will form the laminae of the cortical plate. Throughout the rest of the gestation period until adulthood, the cortical plate gets thicker and more cells migrate from the ventricular zone (97). When proliferation is disrupted, migration is also affected (**Figure 1**). PAE alters proliferation and migration processes (52), affecting neural crest migration and causing cytoskeletal rearrangements. These phenomena destabilize the formation of focal adhesions in cell lineages, reducing their capacity for directional migration. Moreover, the activity of glycogen synthase kinase 3 (GSK3) and cyclin-dependent kinase 5 (cdk5) modulate microtubule-associated protein 1B (MAP1B) phosphorylation, involved in the regulation of microtubules and actin filaments in neurons, needed in migration processes (98). *In vitro*, ethanol inhibits neurite outgrowth by activating GSK3β (99). Conversely, PAE promotes GABAergic interneuron migration by inducing epigenetic alterations in the methylation pattern of the MeCP2-BDNF/DLX5 pathway. MeCP2 regulates the expression of the brain-derived neurotrophic factor (BDNF), a marker of neuronal plasticity and cellular survival known to influence GABAergic interneuron migration (100). MeCP2 has been shown to regulate DLX5 transcription, a transcription factor involved in the migration and maturation of GABAergic interneurons in mouse models (101). The human natural killer-1 (HNK-1) carbohydrate is also used as a biomarker in migration processes studies involving cranial neural crest cells (102). Results indicate reduced levels of HNK-1 in a model of chick embryos exposed to 2% ethanol, which suggests that PAE may disrupt cranial neural crest cell migration.

Long-term effects of PAE on migration have also been evaluated. Miller et al. describe the harmful effects of alcohol on proliferation and migration in rats prenatally exposed to alcohol. The authors found a delay in migration of early and late-generated neurons in rats following PAE between GD 6 and GD 21. Ethanol blocks neuronal migration, probably by leading to a desynchronization of cortical development that interferes with the establishment of a normal neural network (52). Skorput et al. studied the effects of PAE on GABAergic interneurons in mice. They found an increase in BrdU labeling in the medial ganglionic eminence showing an increase in neurogenesis, as well as an increase in parvalbumin-expressing GABAergic interneurons in the medial pre-frontal cortex in adults. These results support the contribution of GABAergic interneuron migration disorders to persistent alterations in cortical development in adulthood (103).

In summary, migration is a set of complex processes regulated by different molecular pathways that are disrupted in several checkpoints when ethanol exposure occurs.

Differentiation

Processes of neuroblast differentiation initiate after neuronal precursors have completed their last division and are ready to migrate to a specific area (104–106). Depending on the fate (brain area) of migration, neuronal precursors may differentiate into neurons, astrocytes, or oligodendrocytes (107). The differentiation of the cerebral cortex implies the formation of laminae in the radial domain from the ventricular zone to the pial surface and the subdivision of functional areas in the tangential domain, in rostrocaudal and mediolateral axes. In this process, the laminar fate is determined by cell-to-cell interactions and cell autonomous restriction on their development (104).

Several proteins, used as biomarkers, are involved in the differentiation processes. Doublecortin (DCX) has been studied in depth as an endogenous marker of immature neurons. The effects of pre-natal chronic ethanol consumption on adult neurogenesis (PND 56) has been assessed in C57BL/6J mice, revealing a decrease of DCX in the hippocampus after PAE (82). Quantification of immature neurons labeled with DCX in mouse was lower in the group of individuals exposed to alcohol in the prenatal period compared to controls. Moreover, DCX levels were lower in males than in females (108). Broadwater et al. obtained similar results after PAE by oral gavage on PND28–48, with decreased DCX levels in the DG of adolescent mice. Furthermore, after interrupting ethanol exposure, reduced levels of differentiated neurons in adulthood were found in rats (109). Elibol-Can et al. observed slight changes in the number of granular cells labeled with DCX in hippocampal DG on PND 30. The authors reported a decrease in the volume of the hippocampus in rats after a daily dose of 6 g/Kg ethanol during second trimester equivalent (110). Likewise, Hamilton et al. studied the long term-effects of single or continuous exposure to alcohol during the third trimester equivalent in mice and the effect of voluntary exercise as a therapy. Mice were exposed to ethanol on PND 7 or PND 5, 7, and 9 and DCX measured in adulthood. No differences in DCX levels were found in ethanol exposed groups. Nevertheless, the group exposed to ethanol during PND 5, 7, and 9 showed alterations in the results obtained in Rotarod and passive avoidance behavioral tests, which measure motor coordination and memory, respectively (111). Conversely, Coleman et al. observed increased levels of DCX after ethanol exposure in the DG in adult PND 7 male mice, but not in females (70).

Long-term effects of PAE have also been studied using other biomarkers. Choi et al. assessed the effects of PAE on BrdU levels in adult mice exposed to ethanol during the two trimester equivalents. No differences in neuronal proliferation nor differentiation were found after evaluating BrdU levels (31). Boehme et al. studied BDNF levels of rats exposed to ethanol during the three trimester equivalents. They found no changes

in BDNF levels of animals exposed to ethanol in the prenatal period. However, increased BDNF levels were observed in groups assigned to voluntary exercise (35). Gil-Mohapel et al. reported increases in NeuroD levels in adult rats exposed to ethanol during the three trimester equivalents. The increase in differentiation processes are probably due to the increase in immature neurons showed in prenatally exposed groups (29). The changes observed in the differentiation processes in adult rodents exposed to ethanol during the prenatal period vary according to the used biomarker. The increase in neuronal differentiation may occur as a compensation of the cellular loss in fetal life.

Responsive element binding protein (CREB) and cAMP signaling is directly correlated to neurogenesis, differentiation, neuronal connectivity, and plasticity (112). Ethanol exposure disrupts the activity of adenylyl cyclase (AC) reducing cAMP/CREB signaling and therefore altering the differentiation processes during neurodevelopment (112). *In vivo* and *in vitro* studies have shown that acute alcohol exposure enhances agonist-stimulated AC catalytic activity, while chronic alcohol exposure produces adaptive changes in AC (113–115). Additionally, GSK3β over-expression in neural cells disrupts CNS maturation and differentiation processes in mouse at PND 60–120 (116).

The glial cell-derived neurotrophic factor (GDNF) is a growth factor necessary for the development, differentiation, proliferation, and function of midbrain dopaminergic neurons. The GDNF signaling pathway is initiated by the binding of GDNF to its co-receptor, GDNF family receptor-α 1 (GFRα1), which leads to the recruitment of the RET receptor tyrosine kinase. The activation of RET promotes the up-regulation of downstream signaling pathways such as ERK1/2 (117) and P13K (118), firing the activity of dopaminergic neurons. Moderate administration of alcohol increases GDNF expression, exerting a protective function against PAE. However, after acute (binge) ethanol exposure in rats, GDNF expression decreases and its protective function diminished (119). A recent study performed in adult rats exposed to alcohol showed a decrease in DNA methylation as the leading cause of GDNF epigenetic changes following alcohol exposure (120).

Alcohol has deleterious effects on astrocytes despite them being less susceptible than neurons to moderate alcohol consumption (121). Glial cell alterations due to PAE lead to changes in neuron-glia interactions, which causes developmental defects of the brain (122). Glial fibrillary acidic protein (GFAP) is a biomarker of mature astrocytes commonly evaluated in differentiation processes during development. *In vitro* studies using primary cultures of astrocytes from 21-day old fetuses show initial increased values of GFAP levels post-ethanol exposure (123), although these GFAP values decrease after 3 weeks (123). GFAP levels in rat neonates have been shown to increase following ethanol exposure in different brain areas, e.g., the hippocampus, cerebellum, and cortex as per different administration routes (124–126). The results in *in vitro* models suggest different effects of ethanol on astrocytes depending on the neurodevelopmental stage. Moreover, some researchers have found increased GFAP expression associated to gliosis after chronic (moderate) and acute low ethanol exposures, in mice (127, 128). These results indicate a high risk of neurodevelopmental disease in acute PAE or heavy drinkers. Conversely, no changes were observed in GFAP expression after low chronic ethanol exposure (127). S100β is a classical biomarker astrocytes, as the expression levels of S100β in these glial cells is very high. During neurite outgrowth, S100β is also secreted by proliferating astrocytes from cortical neurons. The accumulation of this protein in mature glial cells is associated with microtubule network and neurotrophic activity (129). Reduced levels of S100β were reported in mice after ethanol exposure (130), indicating a depletion in the number of proliferating astrocytes and an impairment in the differentiation processes. Otherwise, Sox2 and Oct4 transcription factors regulate the embryonic stem cell pluripotency and the fate of cell lineages by a narrow range of dose-effect (131). Excess of Oct4 compared to Sox2 leads cells to mesoendoderm differentiation, while the other way round, i.e., higher levels of Sox2, promotes neuroectoderm formation. Ethanol exposure of embryonic stem cells in early differentiation generates imbalances between Oct4 and Sox2, which modifies the cellular fate from neuroectoderm to mesoendoderm, altering the formation of the ectoderm lineage and its derived progenitors. The Oct4/Sox2 imbalance is considered one of the leading causes of developmental delay and anatomical disabilities of the CNS observed in FAS phenotypes (131).

Synaptogenesis

The developmental process of synaptogenesis involves biochemical and morphological changes in pre- and post-synaptic components. In rodents, maturation of synaptic connections occurs during the postnatal period (**Figure 1**) (132) and depends on the physicochemical compatibility of pre- and post-synaptic components and the exclusion of inadequate connections. Less harmful effects of alcohol exposure on synaptogenesis have been observed when administered after birth (54, 55), although during neuronal development ethanol seriously alters some mechanisms related to synaptogenesis (54, 55). In a study using a rat model in which individuals were exposed to ethanol 4 weeks before and during pregnancy, the ultrastructural analysis of the cerebellum at PND 7 showed a delayed synaptogenesis and immature appearance of the presynaptic grid (55). PAE affects the expression levels of synaptic proteins such as synapsin 1 and of other proteins of the pre-synaptic (GAP-43, synaptophysin, synaptotagmin) or post-synaptic machinery (MAP 2 and neurogranin). Moreover, ethanol interferes with the function of adhesion molecules such as NCAM (in chick embryo model) (133) and L1 (in mouse model) (134) involved in cell-cell interactions. During the neural processes of migration and morphogenesis, both proteins are involved in the organization and function of synaptic networks, which determine neuronal plasticity. Several studies in animal models (zebrafish) and cell cultures show decreased levels of NCAM after ethanol exposure (135, 136). In other studies, different patterns of NCAM expression were detected according to the developmental stage on which PAE occurs (133) or the NCAM isoform analyzed. For example, the highly sialylated form of NCAM is overexpressed after ethanol exposure but the NCAM 180 and NCAM 140 isoforms appear down-regulated in

a rat model (137). Other studies in animal models (mice and rats) have shown down-regulation of L1 following ethanol exposure (134, 138).

Gliogenesis and Myelination

Glial cells provide nutrients and physical support to neurons and regulate the presence of different proteins and components in the extracellular fluid surrounding neurons and synapses in the brain. They are essential for a normal development and function of the central nervous system (139). Neuroblast migration occurs through a scaffold provided by radial glia (140). Microglia have macrophage functions and astrocytes preserve the ionic and trophic balance of the extracellular medium (141). Oligodendrocytes synthesize myelin, therefore, this cell lineage preserves the myelin sheath and provides trophic support (142). Schwann cells and oligodendrocytes are in charge of the isolation and myelination of neuronal axons (143). Oligodendrocyte progenitor cells proliferate and differentiate into mature oligodendrocytes capable of myelinogenesis (144). Thus, myelination begins later in neurodevelopment than other processes such as proliferation and migration and progresses throughout adolescence in rodents (145, 146). The development of these cell lineages occurs at the same time as neurogenesis in several areas of the central nervous system (141). These lineages are characterized by distinct developmental stages and sequential expression of different developmental biomarkers such as the nerve growth factor (NGF), neurotrophins (NT-3 and NT-4), the brain derived neurotrophic factor (BDNF), and the IGF-1 and IGF-2 factors. The BDNF is one of the most studied neurotrophins. Alcohol alters the levels of BDNF and its receptor tyrosine kinase B (TrkB). PAE induces decreased levels of BDNF in the cortex and in the hippocampus in rats at PND 7–8 (147). Some studies in rats show that TrkB levels decrease in specific brain regions, e.g., in the hippocampus (147, 148) and increase in the cortex (148). The BDNF and its receptor are targets for ethanol damage. Consequently, imbalances between them may contribute to the development of FASD-like phenotypes, even in cases in which the levels of one of them remain unaltered. In general, the up-regulation of these neurotrophic factors show protective effects during development, promoting myelination, cell survival, and neural regeneration in pathological conditions (149).

Lancaster et al. showed that PAE reduces myelinogenesis and its persistence after birth in a rat model (56). Severe impairments in gliosis and a reduction of proteins related to myelin integrity (myelin-associated glycoprotein, myelin basic protein, myelin proteolipid protein, and myelin regulatory factor) was observed in male adult mice exposed to a binge (acute) pattern of PAE during gestation and lactation. This damage was followed by behavioral alterations in executive function and motor coordination (79). These changes could be associated to the behavioral disabilities observed in FASD individuals. It has also been shown that exposure to alcohol activates toll-like receptor 4 signaling pathways (MAPK, NFκB) in a mouse model, leading to an increased expression of pro-inflammatory mediators (COX-2, iNOS, HMGB1) and cytokines. Inflammation processes cause myelinogenesis imbalances, impairments in synaptic links, and activation of the cell death mechanism (150).

Trophic Support

CNS remodeling is a continuous process that not only takes place during development, but also throughout adulthood in response to environmental influences or genetically programmed events. Alcohol alters synaptic plasticity and neural function (151). Several proteins used as biomarkers participate in neural plasticity processes. Histone deacetylase 2 alters the GluN2A/GluN2B balance [the major subunits of functional N-methyl-D-aspartate (NMDA) receptors] through changes in GluN2B expression, which leads to memory-impairing effects (152). The neurotrophin family of proteins includes NGF, BDNF, NT-3, NT-4/5, and NT-6. It is well-known that NGF and BDNF play important roles in PAE and FASD pathogenesis. Various studies have shown that PAE disrupts neurotrophin pathways, thus affecting the organogenesis and development of brain structures in rodents (153, 154). NGF and BDNF exert their biological effects by activating some members of the tropomyosin-related kinase (Trk) family. NGF activates TrkA and BDNF binds to TrkB (155). Stressful events, neurological injuries, or neuroendocrine alterations in rats increase blood levels of NGF (156). Thus, NGF expression and the functional activity of NGF-target cells in the CNS are seriously affected by alcohol consumption. BDNF regulates neural cell survival and differentiation as well as several functions related to neural plasticity such as learning and memory (157). A recent study found that BDNF levels in the pre-frontal cortex were significantly lower in the group of mice treated with ethanol in comparison to the control group (158). The study concluded that the impairment in learning and memory observed in mice exposed to ethanol was associated to changes in BDNF levels. Stragier et al. showed that chronic and moderate alcohol consumption in C57BL/6J mice promotes a chromatin-remodeling process, leading to up-regulation of BDNF signaling. The authors suggest that this epigenetic regulation is an adaptive process to balance cognitive disorders induced by alcohol (159). Another study in mouse observed a reduction in ethanol dependence after BDNF infusion in the pre-frontal cortex (160), evidencing that BDNF levels in specific brain areas play a role in alcohol dependence. Boehme et al. studied the changes produced by voluntary exercise in hippocampal BDNF levels. Ethanol was delivered by intragastric gavage during the three trimester equivalents and individuals had free access to voluntary exercise on a running wheel during adulthood. Results showed increased BDNF levels in young adult females after voluntary exercise (35). Recent studies suggest that matrix metalloproteinase-9 (MMP-9), a Zn $(2)^{+}$ dependent extracellular endopeptidase, participates in neuronal plasticity, specifically in memory and learning (161, 162). Acute and chronic ethanol exposure up-regulates the MMP-9 levels in the brain, particularly in the medial pre-frontal cortex and hippocampus, in rats (163). The vascular endothelial growth factor (VEGF) is involved in the activity, plasticity and survival of microvessels. Mice prenatally exposed to alcohol have reduced cortical vascular density, affected microvascular structure, and altered expression of VEGF and its receptor.

VEGF may prevent microvessel plasticity disorders and death. As a mouse model shows, PAE exerts its deleterious effects on the microvascular network, which suggests that vascular defects contribute to alcohol-induced brain injury (164). *In vitro* studies show that ethanol also alters the expression and function of IGF-I and IGF-II, leading to birth defects such as low head circumference at birth and microcephaly. These insulin-like growth factors are used by the organism as a general signal of cell survival, so that reduced IGF-I or IGF-II signaling by PAE in neurons activates cell death mechanisms by apoptosis or necrosis (165). Other biomarkers such as DYRK1A act as general inhibitors of neural plasticity. Its over-expression in different brain areas due to environmental insults or stress conditions reduces neural plasticity in neurons promoting cognitive problems and intellectual disability (166–168). Recent studies have demonstrated that some DYRK1A inhibitors such as the antioxidant Epigallocatechin gallate (EGCG) improve long-term outcomes related with memory and executive function in individuals with Down syndrome (166–168). Although it is currently under study, the inhibition of DYRK1A could improve the cognitive performance in pathologies associated to the loss of neuronal functions and plasticity, e.g., FASD, Autism, or Down Syndrome (169). Furthermore, EGCG increases NGF expression by downregulation MMP-9. These proteins have been associated with FASD alterations during neurodevelopment (170).

Synaptic Plasticity

Synaptic plasticity is the process through which long-term changes in synaptic communication occur (171).

Fontaine et al. studied the effect of prenatal exposure to ethanol in a rat model during the two trimester equivalent and PND 21–28 on long-term potentiation, long-term depression, and depotentiation in the medial perforant path input to the DG of the hippocampus. Impairment of long-term potentiation was seen in both males and females, while long-term depression was only observed in males. The results suggest that PAE causes sex specific impairment in synaptic plasticity in long-term depression (172). Wong et al. focused their study in the contribution of microglia in synaptic plasticity. Using a third trimester equivalent mice model, ethanol was injected following a binge-drinking pattern. The authors found a deficit in experience-dependent synaptic plasticity in the visual cortex with no correlation to microglial function (173). Shivakumar et al. administered ethanol to mice at PND 7, and showed that ethanol exposure produces epigenetic changes that inhibit the activation of several synaptic plasticity genes. Coadministration of trichostatin A prevents learning and memory disorders in adult mice (174).

PAE negatively affects synaptic plasticity. Epigenetic changes, as well as damage to the microglia, may partially explain synaptic plasticity disorders in FASD models.

Apoptosis

Apoptosis is a critical pathway in fetal neurodevelopment. Programmed cell death systematically removes a large number of neural precursors in embryonic structures formed during development. PAE activates and deregulates cell death mechanisms leading to the loss of cell lineages in the hippocampus, basal ganglia, or cerebellum and disappearance of critical structures in the brain such as the *corpus callosum* (57, 58). The activation of apoptosis is produced by an increase of reactive oxygen species (ROS) generated in ethanol metabolism (see section Oxidative Stress). ROS activate intrinsic and extrinsic apoptotic pathways, reducing the expression and function of the anti-apoptotic proteins Bcl-xL and Bcl-2 in a rat model (175). A study using a mouse model shows that the function of the pro-apoptotic effectors Bak and Bax is directly influenced by alcohol due to alterations in mitochondrial membrane fluidity and dysfunctions in mitochondrial respiration, which leads to the activation of the caspase cascade and subsequent generation of the active form of the effector caspase 3 (176). Consequently, some researchers have developed mitochondrial protective strategies to prevent alcohol-induced damage. Certain molecules, e.g., nicotinamide (177), can stabilize mitochondrial membranes while others, e.g., antioxidants, prevent mitochondrial dysfunction induced by the production of ROS following ethanol exposure, in mouse. In addition, ethanol activates specific cell death pathways. More specifically, ethanol induces the phosphorylation of c-jun N-terminal-kinase, a mitogen-activated protein kinase associated with apoptosis and GDNF may interfere with the activation of the c-jun N-terminal-kinase molecular pathway to prevent ethanol-induced apoptosis. Unlike other neurotoxic substances, ethanol does not interfere with the phosphorylation of the extracellular signal-regulated kinases involved in the regulation of cell survival (178).

PATHOPHYSIOLOGY

There are multiple pathological effects derived from alcohol exposure during fetal development depending on the studied organ, region and cell type, as well as the stage of pregnancy in which the fetus is exposed to ethanol (179). The following sections provide a detailed description of the teratogenic effects of PAE.

Oxidative Stress

Ethanol is metabolized in the liver of adult individuals via the alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) families of enzymes (**Figure 3**), leading to moderate ROS production, e.g., hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH-). ROS are eliminated by endogenous antioxidant mechanisms directed by catalase, superoxide dismutase (SOD) and the antioxidant molecule glutathione (GSH) (180). After a high intake of alcohol, the catalytic activity of ADH and ALDH becomes saturated and an alternative pathway mediated by the cytochrome P450 2E1 enzyme is up-regulated to metabolize ethanol to acetaldehyde, producing high amounts of ROS. ROS-sensing transcription factors, such as the nuclear erythroid 2-related factor 2, activate the oxidative stress response mechanisms when moderate levels of alcohol-derived ROS are present, up-regulating antioxidant enzymes and proteins involved in DNA repair. Imbalances between ROS-producing pathways (following PAE) vs. the endogenous antioxidant and DNA repair mechanisms promotes down-regulation of detoxification pathways (180, 181). The decrease of the

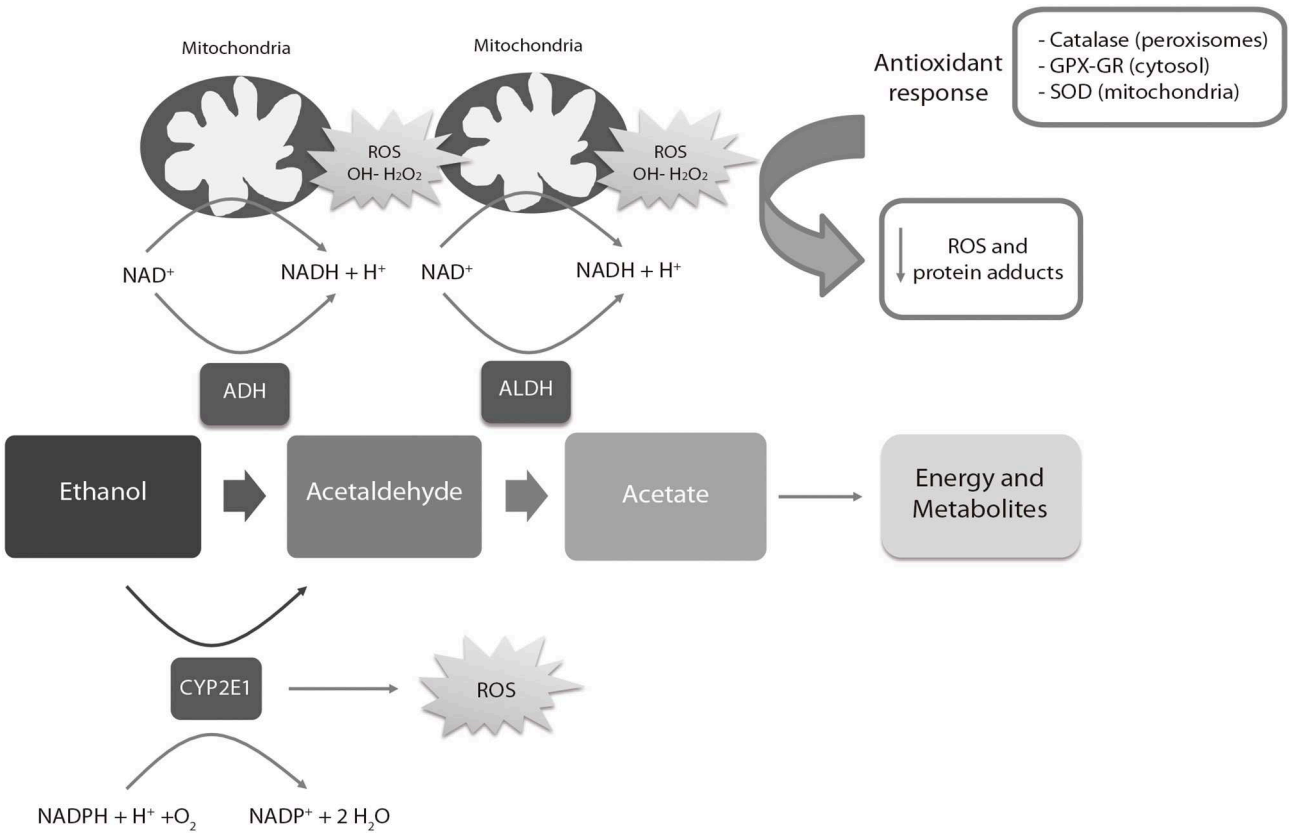

FIGURE 3 | Liver metabolic pathway of alcohol. ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; ROS, reactive oxygen species; SOD, Superoxide Dismutase; GPX, Glutathione peroxidase; GR, Glutathione reductase. The ADH, ALDH enzymes and the P450 2E1 cytochrome participate in the oxidative metabolism of alcohol. The activity of the P450 2E1 cytochrome in alcohol metabolism and the re-oxidation of NADH via the electron transport chain in the mitochondria results in the formation of ROS. Catalase in peroxisomes, SOD in mitochondria and GPX-GR in cytosol are activated by an increase of oxidative stress to reduce the levels of ROS. High amounts of ROS lead to a down-regulation (negative feedback) of the antioxidant response.

antioxidant system affects specific regions of the CNS such as the cerebellum, hippocampus and cortex, as well as the placenta (182, 183). The fetal brain is particularly sensitive to PAE because the ADH isoform expressed in this tissue during development is a class II isoenzyme ADH4. This isoform is less efficient for alcohol catabolism than other isoforms expressed in adults (184). The mechanisms involved in antioxidant response are physiologically downregulated during development (185–188), contributing to brain vulnerability by ethanol. The excess of ethanol also activates the lactate pathway in the fetal liver, generating a deficit of glucose in the bloodstream that affects especially the nervous tissues (189). Imbalances of ROS activate the mechanisms of inflammation (190) mediated by cytokines such as IL-6 or the NLRP3 inflammasome, a multi-protein intracellular complex responsible for processing and secreting the pro-inflammatory cytokines IL-1β and IL-18 (191).

Tissue homeostasis is also affected by ROS (192), causing changes in critical cell functions as signal transduction related to the metabolism of macromolecules (lipids, proteins, RNA, and DNA) (190). AS an example, ROS promote the modification of 8-oxoguanine in DNA during embryogenesis (193, 194), which is corrected by the enzyme oxoguanine glycosylase 1 (195). Calcium homeostasis and protein folding, modification and secretion in endoplasmic reticulum are also altered by ROS, as well as mitochondrial respiration, affecting its morphology and function. Moreover, activation of autophagy, programmed (apoptosis) and non-programmed (necrosis) cell death are also promoted by oxidative stress (192, 196, 197).

Some studies have assessed the long-term consequences of PAE on oxidative stress and the intracellular redox state. Dembele et al. found an relation between continuous administration of PAE with increased levels of oxidative stress in adult rats (PND 90), characterized by high levels of protein carbonyls, lipid peroxides, high expression of SOD, and low levels of GSH (198). Similar results have been reported by other authors, who showed an association between chronic PAE at different concentrations with increased levels of distinct oxidative stress and lipid peroxidation markers in adolescent and adult rodents (199, 200). Chu et al. found a correlation between PAE and apoptotic (p53) and DNA oxidation markers (8-hydroxydeoxyguanosine) in adult rats (200). Brocardo et al. reported depressive and anxiety-like behaviors and high levels of lipid and protein peroxidation

in adult rats (PND60) who were given ethanol throughout the three-trimester equivalents (201). Their findings also indicate an association between voluntary exercise, which increased the endogenous antioxidant pathways in brain, and the reduction of oxidative stress and depressive/anxiety-like behaviors. Similarly, binge drinking model of PAE (GD 17–18) increased the levels of lipid peroxidation and oxidative stress, apoptotic activation via caspase-3 activity, and DNA fragmentation, decreasing antioxidant molecules as GSH (202, 203).

Dysregulation of the Neuroimmune System

Ethanol exposure activates the innate neuroimmune system, causing brain damage and neurodegeneration (150, 204). Alcohol intake triggers the stimulation of microglia and astrocytes, promoting neuroinflammation with the consequent production of pro-inflammatory cytokines and chemokines (e.g., TNF-α, IL-2, IL-6, IL-8, Il-10, IL-1RA, IFN-γ, or MCP-1) (150, 204).

Toll-like receptor 4 and NOD-like receptors have an important function in glial cell stimulation and alcohol-mediated neuroinflammation. Ethanol activates toll-like receptor 4 signaling pathways mediated by NFκB and MAPK, which leads to the up-regulation of cytokines and pro-inflammatory mediators such as HMGB1, COX-2, and iNOS (150). The activation of these inflammation pathways generate severe impairments on synaptic and myelin proteins as well as neural damage (150). Moreover, the increased caspase-3 activity in the prefrontal cortex indicates apoptotic cell death secondary to PAE-related neuroinflammation (205). Regarding myelination and white matter structure, PAE causes neuroimmune changes such as reductions in myelin-associated glycoprotein levels, myelin basic protein and myelin proteolipid protein. Alterations in oligodendrocytes that interfere in the myelination process affecting neural transmission and cognitive development have also been described (79, 205).

Neurotransmitter Disorders

Neuronal cells and neuroanatomical structures are particularly susceptible to toxic compounds during embryonic development, explained by the high sensitivity of the processes during brain formation. Neuronal damage triggers tissue degeneration by inflammation and massive cell death (apoptosis and necrosis) (182, 206). The loss of some progenitor cell lines seriously affects proliferation, migration, and differentiation of mature neuronal cells, essential to configure the distinct regions of the brain and make them functional (207). The high sensitivity to increases in oxidative stress is the main cause of cell death in these parental lineages. This occurs because they lack the molecules and enzymes required for an antioxidant response, i.e., catalase and superoxide dismutase (181). High levels of ROS affect the mitochondrial function in neurons and leads to the activation of apoptosis (208).

In a prenatal ethanol-exposed brain, differentiation from multipotent glial cells to astrocytes occurs prematurely, preventing the correct completion of migration processes (209). These astrocytes are therefore incorrectly located in the brain, causing motor, and cognitive disorders, promoting cell death of these neuronal groups and triggering harmful effects such as the agenesis of the *corpus callosum* (209). Moreover, primary cultures of hippocampal neurons exposed to ethanol show reduced levels of the glucose transporter GLU1 necessary for the correct growth and development of most cell types present in the brain whose main carbon source is glucose (210). Alcohol also alters the levels of neurotransmitters, namely serotonin, dopamine, and glutamate (211). Exposure to alcohol delays serotonin synthesis, blocking the stimulation of astrocytes and the release of the growth factors needed for proper neurodevelopment (212). Ethanol reduces the number of glutamate receptors (NMDA), which in turn affects other neurotransmitter routes generating important alterations in the transmission of nerve signals (213). The acetaldehyde produced by the metabolism of local ethanol in fetal hippocampus inhibits neurosteroid synthesis and blocks NMDA receptors in pyramidal neurons, contributing to synaptic dysfunction associated with severe alcohol intoxication (214). Furthermore, a recent *in vitro* study with rat brain slices exposed to 70 mM ethanol indicates that the combined overexpression of GABA receptors and inhibition of NMDA receptors results in alcohol-induced neurodegeneration during synaptogenesis (215). Consequently, the administration of single doses of an NMDA antagonist in Sprague Dawley rats causes apoptotic neurodegeneration in young animals, although no impairments were identified in adult individuals. Therefore, the NMDA antagonist acts on the CNS in a similar way ethanol does (216).

PAE has neuroapoptotic effects on the up-regulation of GABAergic transmission and deficit of NMDA receptors. The impairment produced by ethanol on developing neurons depends on the specific neural lineage and is age-dependent. A study performed in mice exposed to moderate amounts of alcohol showed that the subunits of NMDA receptors GluN1 and GluN3A are up-regulated after PAE in the DG. The study also found a decrease of GluN2B levels in the synaptic membrane (217).

Epigenetic Modifications

During fetal development, epigenetic mechanisms establish the whole pattern of gene expression for the tissues, organs and cell types that constitute the complete organism. These mechanisms involve the methylation of DNA, modifications of N-terminal tails in histones, and the regulation of micro and non-coding RNA.

In DNA methylation, methyl groups (CH_3), a product of folate metabolism, are added to the cytosines (C) present in the regions known as CpG islands of the DNA helix. This phenomenon is mediated by methyl-transferases (DNMT) and demethylases such as TET2 (218). DNMT3a and 3b set the complete genome expression patterns during fetal development and DNMT1 maintains this pattern in postnatal stages (219). Usually, the clusters of CpG islands match with promoter regions to regulate the expression of the genes involved in a specific pathway or signaling (220). In general, methylation is associated with gene silencing and demethylation with active transcription. Histones regulate the dynamics of chromatin in remodeling processes between heterochromatin, inaccessible to DNA polymerases, and the expanded chromatin (euchromatin) that allows gene expression. Histone structure and function is regulated through

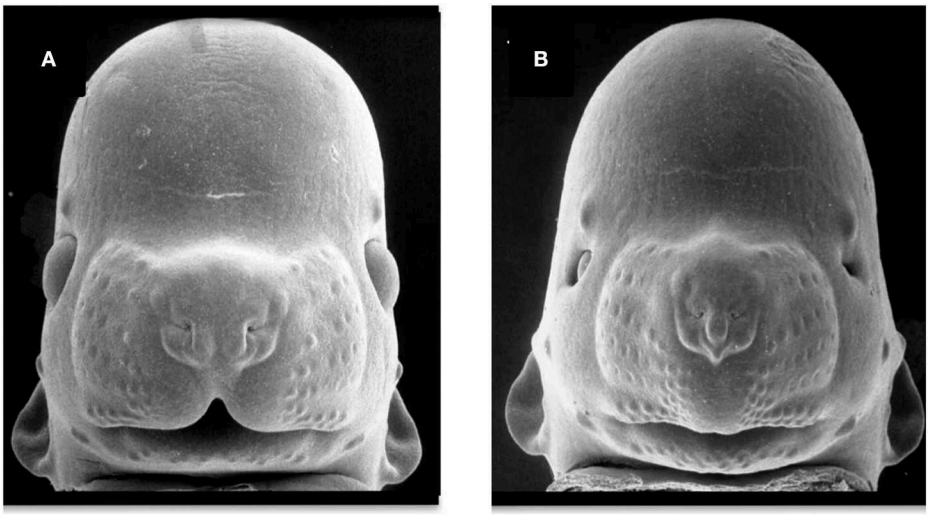

FIGURE 4 | Facial dysmorphology induced by prenatal ethanol exposure. Representative examples of a control animal **(A)** and a fetus severely affected by ethanol exposure **(B)**. FAS-like phenotype **(B)** is defined by microcephaly, short palpebral fissures, thin upper vermillion, and smooth philtrum. FAS-like facial features are induced in the mouse by maternal alcohol exposure on gestational day 7 and 8.5 (equivalent to the third gestational week in humans). Courtesy of Prof. Kathie Sulik, University of North Carolina-Chapel Hill (271) (https://www.teratology.org/primer/fas.asp).

different post-translational modifications in their N-terminal tail such as methylation, acetylation, and phosphorylation, mainly in the amino acids lysine, arginine, and serine. All these reversible modifications are carried out by different enzymes such as kinases, acetyl-transferases or methyl-transferases depending on the requirements of the cell (221). Otherwise, non-coding and microRNAs regulate protein translation and mRNA stability, acting post-transcriptionally as inhibitors of mRNA by direct interaction (222).

Ethanol and ROS can modify the activity of methyltransferases and demethylases, directly affecting the global DNA methylation pattern during development. A recent study performed in mice after PAE found 118 differentially methylated regions (DMRs) related to transcription factor binding sites (223). The pathways affected by these DMRs were epigenetic remodeling, hormonal signaling, metabolism, and immune response, revealing persistent occurrence of these alterations in all developmental stages (223). Demethylation following ethanol exposure causes a decrease in IGF-2 levels *in utero*, generating an important delay in growth and leading to skeletal malformations (224).

PAE influences the activity of enzymes such as histone acetyltransferase and histone deacetylase, which modify the composition of the amino-terminal tails in histones (225). Moreover, the acetylation of histones H3 and H4 has been directly related to alterations in the development of the cerebellum, cardiac defects, and hepatic damage (226, 227). Cantacorps et al. (228) showed that a binge PAE pattern in mice alters histone acetylation (lysine 5 and 12 in histone H4) in the pre-frontal cortex and the hippocampus. These long-term epigenetic modifications are associated with cognitive and behavioral impairments in offspring. Ethanol exposure alters the expression pattern during development, affecting non-coding and microRNAs expression (229). PAE generates a decrease in the expression of miR135, miR9, miR21, and miR355. The absence and deregulation of these microRNAs generate early maturation of the progenitor stem cells and an increase in cell apoptosis, producing severe impairments in fetal brain development (230). The high variability of the described epigenetic changes is associated to the dose of ethanol received, the time of exposure and the gestational stage in which alcohol intake occurs.

DIAGNOSIS OF FASD

PAE results in a wide range of phenotypic manifestations and behavioral deficits in the offspring we describe below.

Craniofacial Anomalies

The key facial features used for a clinical diagnosis of FAS in humans include short palpebral fissures, a thin upper vermilion, and a smooth philtrum (231). Considering previous studies in humans (232, 233), Fang et al. described, for first time in 2009, a validated facial image analysis method based on a multi-angle image classification using micro-video images of mouse embryos. This method, validated later by other researchers, allows discerning between embryos that have been exposed or not to ethanol (232, 233). Rodents provide not only a validated model to study how PAE alters morphogenetic processes, but it also allows making an association between a facial feature alteration and the structure/function in the CNS.

Alcohol exposure during essential periods of embryonic development results in craniofacial dysmorphology (**Figure 4**). Several studies have used chick and murine PAE models to demonstrate the correlation between craniofacial anomalies,

apoptosis induction, and altered migration of neural crest cells (234–236). A series of facial anomalies may present in FASD associated to PAE during the premigratory period of neural crest cells (**Figure 2**). At this stage, ethanol induces calcium transients that activate CaMKII that mediates the loss of transcriptionally active β-catenin, which produces the apoptosis of populations of neural crest cells. Genetic factors play an important role in the vulnerability to alcohol-induced craniofacial dysmorphology. Sonic Hedgehog signaling, platelet-derived growth factor subunit A, Vang-like protein 2, or ribosomal biogenesis genes are of special importance in neural crest development (237). Studies using FASD-like phenotype rodent models, in which dose and timing of ethanol exposure is controlled, show structural alterations in head and face (238, 239) similar to anomalies observed in humans (240).

Several studies have examined the craniofacial anomalies in FASD-like rodent models. According to Godin et al., intraperitoneal administration of two injections of ethanol at 2.9 g/Kg in mice on GD 7 (equivalent to post-fertilization week 3 in humans), generates a series of facial dysmorphologies similar to those seen in FAS children. These defects include median facial cleft, cleft palate, micrognathia, pituitary agenesis, and third ventricular dilatation and heterotopias (33). However, intraperitoneal alcohol exposure of two 25% dosages of ethanol at 2.9 g/Kg delayed to GD 8.5 in mice produces a different pattern of dysmorphologies such as shortening of the palpebral fissures, mild hypoplasia and shortened upper lip, but a preserved philtrum (60). Variations in FAS-like facial phenotypes depend on exposure timing, implying different facial features when considering this variable (**Figure 2**). New techniques for FASD diagnosis include methods to identify potentially at-risk individuals based on the identification of subtle and subclinical facial characteristics (241). Scientists have developed a computerized system for detecting facial characteristics using three-dimensional facial imaging and computer-based dense-surface modeling (241, 242). This approach has been compared against standard dysmorphology physical examination for FAS diagnosis revealing high similarities (243). More recently, new techniques based on MicroCT 3D scan performed on pups prenatally exposed to alcohol have been developed (244). This method showed that craniofacial bones might be a reliable and sensitive indicator of PAE in mouse pups exposed to 4.2% alcohol v/v for 2 weeks before the pregnancy and GD 7–16. The same study also confirmed that the neurocranium (cranial skeleton) is more sensitive to alcohol than the viscerocranium (facial skeleton). Other researchers characterized concurrent face-brain phenotypes in mouse fetuses exposed to two 25% intraperitoneal dosages of ethanol at 2.9 g/Kg on GD 7 or GD 8.5 and using MRI imaging and dense surface modeling-based shape analysis (60). Differences in facial phenotype linked to GD of ethanol exposure were found, being more subtle when the exposure was on GD 8.5. Both phenotypes were associated with unique volumetric and shape abnormalities of the septal region, pituitary, and olfactory bulbs. These findings illustrate the need of increasing the current diagnostic criteria to better capture the full range of facial and brain dysmorphology in FASD.

Brain and Neurobehavioral Deficits

Brain organogenesis is the most severely affected process by alcohol exposure (245) and there is a general consensus in relation to the effects of PAE on the hippocampus, cerebellum, and the *corpus callosum* (246, 247). Important asymmetry of the hippocampus is observed in FAS children, with the left lobe being smaller than the right lobe (8). The cerebellum, associated with balance, coordination and learning capacity, and the anterior part of the vermis develop hypoplasia when exposed to ethanol (7).

The *corpus callosum* is particularly vulnerable to ethanol exposure and, in some cases, may lead to total (agenesis) or partial (hypoplasia) loss of structure (248). The most affected areas of the *corpus callosum* areas are the front (genu) and back (splenium and isthmus), appearing smaller and displaced from the usual spatial location in the brain (6). Basal ganglia is responsible for motor and cognitive abilities, presenting a smaller size in patients with FAS, particularly the area of the caudal nucleus associated to cognitive abilities such as spatial capacity (249). A recent study using three-dimensional surface MRI techniques showed abnormalities in the cortical folding (gyrification) of FASD children. These findings are directly correlated with IQ (250). Future research with MRI techniques to evaluate rodent gyrification may prove to be useful to increase the knowledge on the relationship between cortical development involvement and cognitive disorders in humans.

Broadly, the timing of the ethanol exposure (251) has a clear impact on the CNS and elicits specific brain and behavioral deficits and disorders in motor and cognitive functions (14) (**Figure 2**).

Different standardized tests in rodents have been used to assess FASD-related abnormalities. As the hippocampus is one of the most damages structures when exposed to ethanol, most studies assessed hippocampal function. Spatial learning is commonly evaluated to demonstrate hippocampal disorders (45, 79, 252). Different authors describe long-term motor coordination impairments, learning and memory deficiencies in adult male mice prenatally exposed to alcohol (79), behavioral effects in rats following short-term PAE (253), or depressive-like behaviors in adult rats exposed to ethanol across the three-trimester equivalents (201). To identify the outcomes of gestational alcohol exposure, a summary of behavioral characteristics after alcohol exposure is needed. **Table 2** summarizes the standardized behavioral tests used in rodents to analyze the harmful effects of PAE.

Fetal Growth Restriction

Ethanol interference with maternal nutrition may differ. As a source of energy, alcohol blocks the absorption of other nutrients, including proteins, and hinders intestinal transport of essential nutrients. Due to its effects on liver, alcohol causes metabolic and nutrient utilization alterations. PAE causes maternal nutritional deficiencies that result in fetal growth deficiencies (26).

PAE also impairs placental angiogenesis (269) and consequently fetal growth restriction (FGR) (270). The growth curves defined by Dilworth et al. are a useful tool to define the frequency distribution of mouse weight. Any fetus with a weight below the fifth centile was considered growth restricted (68).

TABLE 2 | Standardized experimental methodologies for assessing behavioral effects of prenatal alcohol exposure in murine models.

Skills affected by PAE	Disorders	Behavioral test	Description
Motor skills Cerebellum (Purkinje cells) (254)	Motor hyperactivity, poor motor coordination, altered accuracy of saccadic eye movements, and deficits in postural balance are impaired motor skills observed in individuals exposed to ethanol during early life. PAE-related motor deficits are more apparent in early life than in adulthood.	The rotarod (79)	For this test, a bar that can rotate at an accelerated or a fixed speed is used. The latency of the fall of the rodent placed in the bar is measured. Measurements provide an idea of motor coordination.
		Swimming test (255)	It consists of a Perspex tank where rodents are placed and must swim to an escape platform. The sequence is recorded and later analyzed to assess the latency to reach the platform and the number of fore and hind limb strokes.
		Raised beam test	Rodents are placed on the bar and their ability to cross it is measured along with paw slips and traverse time. It provides an indication of their balance.
		Footprint analysis	After their paws are painted or dipped in ink, rodents leave a trail of footprints when they walk or run along a corridor to a goal box. Measurements of stride length, base width, and fore and hind paws overlap give an indication of gait. Automated versions of the task use video processing of footage taken from below the rodents.
Learning and memory Hippocampus (dentate gyrus) (256, 257)	Hippocampal cell loss, altered neuronal morphology, decreased synaptic density, and reduced trophic support.	Simple maze task (258, 259)	A T-maze (or the variant Y-maze) is a simple maze used in animal cognition experiments. It is shaped like the letter T (or Y), providing the subject, typically a rodent, with a straightforward choice. T-mazes are used to study how rodents function with memory and spatial learning by applying different stimuli. The different tasks, such as left-right discrimination and forced alternation, are mainly used with rodents to test reference and working memory.
		Morris water maze (71, 252)	MWM is a test of spatial learning for rodents that relies on distal cues to navigate from a start point around the perimeter of an open swimming box to locate a submerged escape platform. The test allows measuring spatial learning and reference memory.
		Fear conditioning (260)	This test is a form of Pavlovian learning based on the conditioning of an innate response to fear consisting in a complete lack of movements. During an initial phase, the animal is exposed to a conditioned stimulus paired with an aversive experience (unconditioned stimulus). The test measures the fear response in mice replaced in the same location with and without the previous stimulus.
		Object recognition (79, 261)	In an initial session, the rodent is presented with two similar objects. One of the objects is replaced by a new object in the second session. The test measures the amount of time taken to explore the new object.

(Continued)

TABLE 1 | Continued

Skills affected by PAE	Disorders	Behavioral test	Description
Executive function frontal cortex and extra-frontal cortex (262)	Disorders in cognitive control of behavior, including basic cognitive processes such as attentional control, cognitive inhibition, inhibitory control, working memory, and cognitive flexibility.	Passive avoidance (263)	Mice learn to inhibit the natural tendency to explore new environments where a negative stimulus was previously obtained. The test consists of a chamber divided in two compartments separated by a gate. Animals are allowed to explore both compartments in the initial phase. In the following phase, they obtain a negative stimulus in one of the compartments. Animals will learn to associate certain properties of the chamber with the negative stimulus. The test measures the latency to cross the gate between the two compartments when the animal is placed in the compartment where no aversive stimulus were obtained.
		Simple maze task (258, 259)	See description in learning and memory.
		Morris water maze (71, 252, 264)	See description in learning and memory.
		Prepulse inhibition (265)	PPI is a neurological phenomenon in which a soft pre-stimulus (pre-pulse) inhibits the reaction of the animal to a subsequent strong stimulus (pulse) often using the startle reflex. Stimuli may be acoustic, tactile, or luminous.
Social behavior (251)	Poor social skills and inappropriate social interactions.	Observation (251)	Feeding difficulties in neonates and lack of parental care. Aggressive behaviors in adults and reversed behaviors between males and females.
Affective behavior (201)	Anxiety- and depressive-like behaviors (201)	Elevated-plus maze (266)	The device is made up of open arms and closed arms, crossed in the middle perpendicularly to each other. Mice have access to all of the arms. The number of entries into the open arms and the time spent in each arm are used as a measure of anxiety-like behavior.
		Forced-swim test (267)	The test is based on the assumption that an animal placed in a container filled with water will try to escape. However, it will eventually exhibit immobility that may be considered a measure of depressive-like behavior.
Olfaction (253)	Injury of the olfactory circuits.	Classical conditioning tasks (268)	An odor is paired with a tempting or aversive stimulus and the response of the animal to the odor is followed by the tester.

PAE, prenatal alcohol exposure.

Middaugh et al. characterized the impaired growth of C57BL/6 mice prenatally exposed to alcohol (271) showing the influence of alcohol on fetal growth when administered in the second and third trimester equivalents (271, 272).

Other authors have described the effects of ethanol on trophoblasts and placental permeability. Gundogan showed an altered branching morphogenesis in the labyrinthine zone and the suppression of invasive trophoblastic precursors. This altered process compromised fetal growth and placentation in a dose-response manner (69). The permeability inducer VEGF was up-regulated in mouse placenta after acute alcohol exposure. Permeability was also affected by altered structures in the barriers that separate feto-maternal blood circulation (273). Therefore, altered growth factors in conjunction with malformations of the placental barrier may contribute to placental malfunction and permeability alterations in the feto-maternal barrier.

Biomarkers for PAE

A biomarker is objectively measured and assessed as an indicator of a normal biological or pathogenic process, or a pharmacologic response to a therapeutic intervention (274). Here we will use the term biomarker as the molecular or genetic indicator that

identifies prenatal exposure to ethanol. In murine models, the researcher controls the dose and timing of alcohol exposure and eliminates other variables (e.g., other drugs) that may skew the results. This, and the possibility of getting multiple matrices from the rodents, will allow to obtain appropriate biomarkers for PAE detection.

Some authors have demonstrated a significant decrease of alpha-fetoprotein, a perinatal stress biomarker, in the amniotic fluid of B6J litters exposed to alcohol on Day 8 of gestation, although no differences were found in the B6N substrain (275). Other biomarkers, such as fatty acid ethyl esters (FAEEs), a product of non-oxidative ethanol metabolism and a validated biomarker for PAE, have been detected in mouse heart, liver, placenta, and fetal tissues, 1 h after maternal ethanol exposure. FAEEs were shown to persist for at least seven days in the placenta of the mice and at least 14 days in fetal rat organs (276). Unfortunately, FAEEs cannot be measured in neonatal rodents due to the lack of neonatal hair. By contrast, guinea pigs allow a good approximation since they are born with hair (277). Some authors have shown that FAEE concentrations in exposed offspring samples taken at PND 1 were more than 15-fold higher than their control counterparts (278).

On the other hand, changes in selective neurotransmitters from fetal brains of prenatal alcohol-treated C57BL/6 mice were also observed. Authors showed significant reductions in dopamine, norepinephrine, epinephrine, serotonin, and GABA levels in E13 fetal brains. These results would explain the main causes of abnormalities in brain function and behavior found in fetal alcohol spectrum disorders (279).

In recent years, epigenetic studies in rodents highlight the potential of DNA methylation, histone modification, or non-coding RNA species as biomarkers of PAE. Most of these studies have evaluated general changes for each epigenetic modification. DNA methylation has been the most analyzed marker for PAE-induced epigenetic dysregulation, showing that PAE promotes a global pattern of hypomethylation on fetal DNA during pregnancy affecting critical genes such as *bdnf* (280). Haycock et al. demonstrated that genomic imprinting was also deregulated by PAE, in mouse embryos (281). Low levels of *igf2* expression correlates with PAE due to a specific CpG hypomethylation found in its promoter region (224). *Pomc* expression in neurons, related to stress response, is also reduced by CpG hypermethylation in its promoter (282). The authors suggest that this alteration can be transmitted to offspring, raising the hypothesis that the effect of PAE not only occurs when the fetus is exposed to alcohol but also throughout its whole life and future progeny. Hence, the use of these epigenetic changes using CpG methylations as biomarkers of PAE may be a challenge to consider.

Regarding histone modifications, several studies have found PAE-specific alterations on PAE on H3K9ac, H3K4me2, H3K27me3, and H3K9me2, particularly in the brain. These changes are related to alcohol response mechanisms, e.g., H3K9ac, which has been shown to increase after PAE down-regulates genes related to alcohol response (283, 284). Moreover, the increase of H3K4me2 promotes the up-regulation of genes related to alcohol response (284). A general increase of H3K27me2 was observed in the brain in response to PAE (285), more specifically in the hippocampus and neocortex. H3K9me2 also increased after alcohol exposure suggesting persistent alterations in the expression pattern for a long period, and as such has it being considered as a potential biomarker of PAE (286).

Alterations in non-coding RNA expression following PAE have been assessed in rodents. Results show that PAE causes the suppression of several miRNA such as miR-21 and miR335 in fetal neuronal and progenitor stem cells (230, 287). PAE also suppresses the expression of miR-9 and miR-153 and increases the levels of miR-10a during pregnancy (287, 288). Similar results have been reported in zebrafish after PAE (289) and in a rodent model of alcohol use disorder (290). However, due to the heterogeneity, the low reproducibility and the lack of correlations in the results from the different studies, no consensus has been reached for non-coding RNA, showing the complexity of epigenetic interactions when they are altered by PAE.

Animal models play an important role in the identification and validation of new candidate biomarkers, e.g., selective neurotrasmitters, igf1, igf2, and miRNA. In humans, only the direct biomarkers fatty acid FAEEs, ethyl glucuronide, ethyl sulfate, and phosphatidylethanol in biological matrices are validated to detect PAE (291). The low levels of *igf2* expression after PAE (224) in mouse are in line with the results of a recent publication that compared the levels of IGF I and IGF II in the FASD pediatric population with children objectively non-exposed to ethanol (292). These results highlight the potential use of IGF-I and IGF-II as surrogate biomarkers of the damage induced by PAE. Furthermore, BDNF levels in rodent models are known to be disrupted during acute/chronic and prenatal alcohol consumption (293). Thus, changes of BDNF levels in the meconium, cord blood, or in the mother's/infant's serum are used as potential biomarkers of PAE in humans based on rodents results (293, 294). Moreover, magnetic resonance spectroscopy studies in rodents have shown that neurotransmitter biomarkers of FAS including choline, acetyl choline, N-acetyl aspartate, and glutamate, a precursor for the synthesis of GABA, are significantly reduced in FAS (295). Reduced levels of glutamate, taurine, and N-methyl D-aspartic acid receptor have also been observed in FAS children (296, 297). PAE alters the methionine-homocysteine pathway in rodents and humans. Thus, s-adenosylmethionine, which acts as donor of methyl groups to DNA methylases, may be a promising clinical biomarker of FASD (298).

DISCUSSION

FASD is a growing problem in our society. Diagnostic difficulties, limited knowledge on the underlying mechanisms of ethanol toxicity, and absence of effective strategies to treat this pathology is a serious medical matter. There is a wide range of FASD-like animal models, and researchers must be very precise and choose the one that suits best the objective of the study. Invertebrates and simple vertebrates allow alcohol exposure and the assessment of physical malformations and simple behaviors at different developmental periods (299). However,

when studying brain structures or complex behaviors, mammals offer significant advantages compared to the above mentioned models (19). Further insights on FASD is possible with murine models as they allow evaluating the specificity of dose-dependent alcohol teratogenic effects, the timing and developmental stage of fetuses, brain structures, and complex behaviors. Rodents are useful for exploring promising treatments that may help minimize the effects of PAE. C57BL/6 mice are one of the most commonly used mammals in FASD research because they are easy to handle when searching for malformations or complex behaviors after alcohol exposure. A wide range of precise methodologies and experimental protocols have been performed using these animal models. In this review, we have described and compared these protocols to provide a framework that allows researchers to make the correct choice of animal model in their research project.

Rodents and humans have similar stages of brain development, differing in birth timing. The third trimester equivalent in rodents is postnatal. It seems clear that facial dysmorphology appears when ethanol exposure occurs during first trimester equivalent (33, 60, 236, 300). However, it is difficult to establish the optimal period for ethanol exposure when brain and behavioral alterations are explored, because brain developmental processes occur continuously throughout the second and third trimester equivalent. The criteria to choose an appropriate FAS-like model will depend on the experimental design and research questions, for which different patterns of alcohol exposure, tissue analysis, molecular mechanisms, or cell types assessment will be needed.

The pattern of ethanol exposure and dosages are important parameters. Some studies support the hypothesis that a lower daily dose of alcohol administered in a binge-like pattern results in lower brain weight and greater cell loss than a higher daily dose administered in a non-binge-like pattern, as binge-like patterns lead to higher BAC peaks (24, 301).

It is essential to identify FASD manifestations in the experimental models to reach the objectives of the research. Different biomarkers are involved in the distinct stages of brain development (summarized in **Figure 1**), which can be of help when studying the effects of ethanol intake during pregnancy. FAS biomarkers are useful to evaluate the effects of experimental therapies. There is a wide spectrum of techniques to assess facial dysmorphology, e.g., the validated facial image analysis method based on a multi-angle image classification using micro-video images of mouse embryos (233), and other experimental novel techniques based on MicroCT 3D (244). To assess neurodevelopment and behavior of FASD-like rodent models, several standardized behavioral measurements have been described (302, 303) (summarized in **Table 2**), which allow evaluating the different spheres affected by PAE. When choosing the most appropriate test, the skills the researcher wants to analyze must be taken into consideration. The Morris water maze allows an accurate evaluation of a set of cognitive and motor behaviors affected by PAE. It is necessary to underline the existence of other useful alternatives to evaluate these behaviors.

Fetal growth is impaired by the harmful effects alcohol exerts on angiogenesis. Fetal growth restriction may be evaluated through standardized fetal measurements in defined frequency distribution curves (68), or by assessing the placenta using biomarkers and histopathological analysis.

The use of animal models results essential in pre-clinical studies to evaluate the toxicity of potential pharmacological tools. Animal models also provide an insight into the molecular mechanisms altered by alcohol as per the developmental timing of exposure, pattern of exposure, and dosage. Thus, any experimental breakthrough may be directly applied in clinical care to improve the diagnosis and treatment of FASD patients.

This review contains updated information on FAS-like model in rodents, aiming to be a useful reference for researchers working with FASD-like murine models. This is not a systematic review, although we have performed an in-depth narrative review on the topic. We have reviewed methodologies and protocols as per the objectives of the study in order to obtain robust conclusions for future studies. This work will facilitate decision-making when designing a FASD experiment in rodents by exploring and summarizing the currently available information on prenatal alcohol effects in each pregnancy equivalent trimester.

Additional knowledge on cellular, biochemical, genetic and molecular mechanisms, and pathways altered by PAE is necessary. This will allow further experimental research with murine models aiming to improve diagnostic strategies, prevention and treatment for alcohol-related problems.

AUTHOR CONTRIBUTIONS

LA and VA-F drafted the initial manuscript and conceptualized the topics, tables, and figures. MG-R, OG-A, VA-F, and LM conceptualized, designed, and coordinated the review. EN-T, LA, RA-L, and MS-D revised the different versions of the manuscript and prepared the figures and tables. All authors critically reviewed the manuscript and approved the final version for publication.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

APPENDIX III

Other articles published during the development of this thesis

- Navarro-Tapia E, **Almeida-Toledano L**, Sebastiani G, Serra-Delgado M, García-Algar Ó, Andreu-Fernández V. Effects of Microbiota Imbalance in Anxiety and Eating Disorders: Probiotics as Novel Therapeutic Approaches. *Int J Mol Sci*. 2021 Feb 26;22(5):2351. doi: 10.3390/ijms22052351. PMID: 33652962; PMCID: PMC7956573.

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