universidad de león

PhD THESIS

GUT MICROBIOTA MODULATION AS AN ALTERNATIVE IN THE MANAGEMENT OF NON-ALCOHOLIC FATTY LIVER DISEASE AND OBESITY

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A mamá, papá y Mario

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Hoy es momento de echar la vista atrás y hacer balance. Hace nueve años que cogí las maletas para empezar aquello que me ilusionaba en una nueva ciudad: León. Lo que no sabía entonces es que hoy vería ese lugar como mi segundo hogar. Su catedral, su barrio Romántico, sus bares y tapas, sus montañas o su ambiente eterno dan a León esa magia que no todos los lugares tienen. Aunque, realmente, la responsabilidad de que León sea hogar es de todas aquellas personas que han hecho, de una forma u otra, que estos nueve años hayan sido inolvidables.

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volver a coincidir en el trabajo, compañero (en comidas y cenas, estoy segura de que lo haremos).

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*Moltes gràcies, **Alba**. Intentaré escriure això en català. Gràcies pel suport incondicional i l'ajuda d'algú que va més enllà de ser un supervisor. Des del primer dia em vas obrir les portes de la teva casa i tant Mario com jo estem eternament agraïts. Gràcies per tot el treball i el temps que vas emprar amb mi, per ensenyar-me tot allò que era a la teva mà, professional i personal, per la pressió per a poder créixer. Gràcies per deixar-me conèixer a aquest petit terratrèmol del qual només puc dir coses meravelloses: Biel, el meu suec-català. No sabeu com us trobo a faltar, estic segura que ens veurem aviat.*

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La ciencia, muchacho, está hecha de errores, pero de errores útiles de cometer, pues poco a poco, conducen a la verdad.

~ Julio Verne ~

LIST OF CONTENT

SCIENTIFIC CONTRIBUTIONS

The current PhD Thesis entitled "*Gut microbiota modulation as an alternative in the management of non-alcoholic fatty liver disease and obesity*" is presented by compendium of publications, and includes a total of three published articles whose information is detailed below:

» **Long-term effects of bariatric surgery on gut microbiota composition and faecal metabolome related to obesity remission.**

Juárez-Fernández M, Román-Sagüillo S, Porras D, García-Mediavilla MV, Linares P, Ballesteros-Pomar MD, Urioste-Fondo A, Álvarez-Cuenllas B, González-Gallego J, Sánchez-Campos S, Jorquera F, Nistal E.

Nutrients (2021) 13(8):2519. doi: https://doi.org/10.3390/nu13082519.

JCR Impact Factor (IF) (2021): 6.706 | Rank in Nutrition & Dietetics: 15/90 (Q1; D2)

» **The synbiotic combination of *Akkermansia muciniphila* and quercetin ameliorates early obesity and NAFLD through gut microbiota reshaping and bile acid metabolism modulation.**

Juárez-Fernández M, Porras D, Petrov P, García-Mediavilla MV, Román-Sagüillo S, Soluyanova P, Martínez-Flórez S, González-Gallego J, Nistal E, Jover R, Sánchez-Campos S.

Antioxidants (2021) 10(12):2001. doi: https://doi.org/10.3390/antiox10122001.

JCR IF (2021): 7.675 | Rank in Food Science & Technology: 12/144 (Q1; D1)

» **Enhanced mitochondrial activity reshapes a gut microbiota profile that delays NASH progression.**

Juárez-Fernández M*, Goikoetxea-Usandizaga N*, Porras D, García-Mediavilla MV, Bravo M, Serrano-Maciá M, Simón J, C. Delgado T, Lachiondo-Ortega S, Martínez-Flórez S, Lorenzo O, Rincón M, Varela-Rey M, Abecia L, Rodríguez H, Anguita J, Nistal E, Martínez-Chantar ML, Sánchez-Campos, S.

Hepatology (2022) 00:1-16. doi: https://doi.org/10.1002/hep.32705.

JCR IF (2022): 17.298 | Rank in Gastroenterology & Hepatology: 6/93 (Q1; D1)

Moreover, the results included in the current PhD Thesis have been presented in the following national and international congresses:

1. **EMBO Workshop: Energy balance in metabolic disorders.** Málaga (Spain), 03/10/2022 – 06/10/2022. Poster presentation.
'Gut microbiota profile associated to an enhanced mitochondrial activity counteracts NASH progression'. Juárez-Fernández M*, Goikoetxea-Usandizaga N*, Porras D, García-Mediavilla MV, Bravo M, Serrano-Maciá M, Simón J, Delgado T, Lachiondo-Ortega S, Martínez-Flórez S, Lorenzo O, Rincón M, Varela-Rey M, González-Gallego J, Abecia L, Rodríguez H, Anguita J, Nistal E, Martínez-Chantar M, Sánchez-Campos S.

2. **The International Liver Congress 2022 (ILC).** London (United Kingdom), 22/06/2022 – 26/06/2022. Poster presentation.
'Mitochondrial hyperactivation determines a transferable protective gut microbiota profile in metabolic-associated fatty liver disease development'. Juárez-Fernández M, Goikoetxea-Usandizaga N, Porras D, García-Mediavilla MV, Rodríguez H, Nistal E, Martínez-Flórez S, Rincón M, Varela-Rey M, González-Gallego J, Abecia L, Anguita J, Martinez-Chantar M, Sánchez-Campos S. *Journal of Hepatology*, 77: S177-S178. doi: 10.1016/S0168-8278(22)00729-2.

3. **3rd World of Microbiome Conference: Digestive & Metabolic Health.** Vienna (Austria), 28/04/2022 – 30/04/2022. Poster presentation.
'Protective effect on obesity and non-alcoholic fatty liver disease of quercetin and A. muciniphila combination modifying inflammatory status, lipid metabolism and gut microbiota profile'. Román-Sagüillo S, Juárez-Fernández M, Porras D, García-Mediavilla MV, Martínez-Flórez S, Petrov P, Jover R, González-Gallego J, Nistal E, Sánchez-Campos S.

4. **3rd World of Microbiome Conference: Digestive & Metabolic Health.** Vienna (Austria), 28/04/2022 – 30/04/2022. Poster presentation.
'Mitochondrial hyperativation determines a specific microbiota profile conferring a transferable protective effect on non-alcoholic fatty liver disease progression'. Román-Sagüillo S, Juárez-Fernández M, Goikoetxea-Usandizaga N, Porras D, García-Mediavilla

MV, Rodríguez H, Martínez-Flórez S, Rincón M, Varela-Rey M, González-Gallego J, Abecia L, Anguita J, Nistal E, Martínez-Chantar M*, Sánchez-Campos S*.

5. 3rd International Conference on Fatty Liver (ICFL). Vienna (Austria), 28/04/2022 – 30/04/2022. Poster presentation.
'Mitochondrial hyperactivation determines a gut microbiota profile with a transferable protective effect against metabolic associated fatty liver disease'. Juárez-Fernández M*, Goikoetxea-Usandizaga N*, Porras D, García-Mediavilla MV, Rodríguez H, Martínez-Flórez S, Rincón M, Varela-Rey M, González-Gallego J, Abecia L, Anguita J, Nistal E, Martínez-Chantar M*, Sánchez-Campos S*.

6. 3rd International Conference on Fatty Liver (ICFL). Vienna (Austria), 28/04/2022 – 30/04/2022. Poster presentation.
'Inflammatory status, lipid metabolism and gut microbiota as targets of the protective effect of quercetin and A. muciniphila combination on obesity and MAFLD'. Juárez-Fernández M, Porras D, García-Mediavilla MV, Román-Sagüillo S, Martínez-Flórez S, Petrov P, Jover R, González-Gallego J, Nistal E, Sánchez-Campos S.

7. The Liver Meeting. American Association for the Study of Liver Diseases (AASLD). Online congress, 12/11/2021 – 15/11/2021. Poster presentation.
'Anti-fibrotic and anti-inflammatory effects of MCJ deficiency against NAFLD in conventional and transplanted germ-free mice involve gut microbiota modulation'. Porras D*, Goikoetxea-Usandizaga N*, García-Mediavilla MV, Rodríguez H, Nistal E, Juárez-Fernández M, Martínez-Flórez S, Rincón M, Varela-Rey M, González-Gallego J, Abecia L, Anguita J, Martínez-Chantar M, Sánchez-Campos S. *Hepatology*, 74: 1055A.

8. The Liver Meeting. American Association for the Study of Liver Diseases (AASLD). Online congress, 12/11/2021 – 15/11/2021. Poster presentation.
Juárez-Fernández M, Román-Sagüillo S, Porras D, García-Mediavilla MV, Linares P, Ballesteros-Pomar M, Urioste-Fondo A, Álvarez-Cuenllas B, González-Gallego J, Sánchez-Campos S, Nistal E, Jorquera F. *Hepatology*, 74: 1110A.

9. XV Jornadas CIBER Enfermedades Hepáticas y Digestivas. Barcelona (Spain), 8/11/2021 – 9/11/2021. Poster presentation.
'Gut microbiota profile associated to mitocondrial hyperactivation has a transferable protective effect against MAFLD'. Goikoetxea-Usandizaga N*, Juárez-Fernández M*, García-Mediavilla MV, Rodríguez H, Nistal E, Porras D, Martínez-Flórez S, Rincón M, Varela-Rey M, González-Gallego J, Abecia L, Anguita J, Martínez-Chantar M*, Sánchez-Campos S*.

10. XV Jornadas CIBER Enfermedades Hepáticas y Digestivas. Barcelona (Spain), 8/11/2021 – 9/11/2021. Poster presentation.
'Long-term metagenome and metabolome signatura of bariatric surgery'. Román-Sagüillo S, Juárez-Fernández M, Porras D, Linares P, Andrés-Amo A, Ballesteros-Pomar M, Urioste-Fondo A, Álvarez-Cuenllas B, González-Gallego J, Sánchez-Campos S, Nistal E, Jorquera F, García-Mediavilla MV.

11. XV Jornadas CIBER Enfermedades Hepáticas y Digestivas. Barcelona (Spain), 8/11/2021 – 9/11/2021. Poster presentation.
'The potential synbiotic based on quercetin and A. muciniphila as a feasible strategy facing obesity and MAFLD'. Juárez-Fernández M, Román-Sagüillo S, Petrov P, Porras D, García-Mediavilla MV, Soluyanova P, Andrés-Amo S, Martínez-Flórez S, González-Gallego J, Nistal E, Jover R, Sánchez-Campos S.

12. 3ª Reunión de Hepatología Traslacional. Enfermedad hepática metabólica: del hígado graso a la cirrosis y sus complicaciones. Asociación Española para el Estudio del Hígado (AAEH). Alicante (Spain), 22/10/2021 – 23/10/2021. Poster presentation.
'Estado inflamatorio, metabolismo lipídico y microbiota intestinal como dianas del efecto protector de la combinación de quercetina y A. muciniphila en el desarrollo de obesidad y EHMG'. Román-Sagüillo S, Juárez-Fernández M, Porras D, García-Mediavilla MV, Martínez-Flórez S, Petrov P, Jover R, González-Gallego J, Nistal E, Sánchez-Campos S.

13. 3ª Reunión de Hepatología Traslacional. Enfermedad hepática metabólica: del hígado graso a la cirrosis y sus complicaciones. Asociación Española para el

Estudio del Hígado (AAEH). Alicante (Spain), 22/10/2021 – 23/10/2021. Poster presentation.

'La hiperactivación mitocondrial determina un perfil de microbiota intestinal con efecto protector transferible en el desarrollo de EHMG'. Juárez-Fernández M*, Goikoetxea-Usandizaga N*, Porras D, García-Mediavilla MV, Rodríguez H, Martínez-Flórez S, Rincón M, Varela-Rey M, González-Gallego J, Abecia L, Anguita J, Nistal E, Martínez-Chantar M*, Sánchez-Campos S*.

14. 46 Congreso de la Asociación Española para el Estudio del Hígado (AEEH). Madrid (Spain), 14/06/2021 – 16/06/2021. Oral communication.

'Microbiota asociada a una hiperactividad mitocondrial revierte la enfermedad hepática por depósito de grasa'. Juárez-Fernández M*, Goikoetxea-Usandizaga N*, Porras D, García-Mediavilla MV, Rodríguez H, Nistal E, Martínez-Flórez S, Rincón M, Varela-Rey M, González-Gallego J, Abecia L, Anguita J, Martínez-Chantar M*, Sánchez-Campos S*. *Gastroenterología y Hepatología,* 2021, 44 (Espec Congr 2):6-10.

15. 46 Congreso de la Asociación Española para el Estudio del Hígado (AEEH). Madrid (Spain), 14/06/2021 – 16/06/2021. Poster presentation.

'El simbiótico formado por Akkermansia muciniphila y quercetina mejora la obesidad temprana y NAFLD a través de la modulación de la microbiota intestinal y el metabolismo de los ácidos biliares'. Juárez-Fernández M, Porras D, Petrov P, García-Mediavilla MV, Román-Sagüillo S, Soluyanova P, Martínez-Flórez S, González-Gallego J, Nistal E, Jover R, Sánchez-Campos S. *Gastroenterología y Hepatología,* 2021, 44 (Espec Congr 2):32-125.

16. 46 Congreso de la Asociación Española para el Estudio del Hígado (AEEH). Madrid (Spain), 14/06/2021 – 16/06/2021. Poster presentation.

'Efectos beneficiosos de la cirugía bariátrica sobre la composición y funcionalidad de la microbiota intestinal en pacientes con obesidad extrema'. Román-Sagüillo S, Juárez-Fernández M, Porras D, García-Mediavilla MV, Linares, Ballesteros-Pomar M, Urioste-Fondo A, Álvarez-Cuenllas B, González-Gallego J, Sánchez-Campos S, Jorquera F, Nistal E. *Gastroenterología y Hepatología*, 2021, 44 (Espec Congr 2):32-125.

17. **ECOONLINE 2021. 28th European Congres son Obesity. European Association for the Study of Obesity (EASO).** Online congress, 10/05/2021-13/05/2021. Oral communication.
'Reshaping gut microbiota composition and bile acid signalling as beneficial mechanisms in the management of early obesity and fatty liver with Akkermansia muciniphila and quercetin supplementation'. Juárez-Fernández M, Porras D, Petrov P, García-Mediavilla MV, Román-Sagüillo S, Martínez-Flórez S, González-Gallego J, Nistal E, Jover R, Sánchez-Campos S. *Obesity Facts* 2021, 15 (suppl.1):21-22.

18. **ECOONLINE 2021. 28th European Congress on Obesity. European Association for the Study of Obesity (EASO).** Online congress, 10/05/2021-13/05/2021. Oral communication.
'Bariatric surgery impacts on gut microbiota composition and faecal metabolome profile in patients with morbid obesity'. Román-Sagüillo S, Juárez-Fernández M, Porras D, García-Mediavilla MV, Linares P, Ballesteros-Pomar M, Urioste-Fondo A, Álvarez-Cuenllas B, González-Gallego J, Sánchez-Campos S, Jorquera F, Nistal E. *Obesity Facts* 2021, 15 (suppl. 1):58-59.

19. **VII Jornadas de Investigadoras de Castilla y León.** Valladolid (Spain), 15/04/2021-16/04/2021. Oral communication.
'La modulación de la microbiota intestinal: estrategia terapéutica para el manejo de obesidad y enfermedad de hígado graso no alcohólico (NAFLD)'. Juárez-Fernández M, Porras D, Petrov P, García-Mediavilla MV, Román-Sagüillo S, Martínez-Flórez S, González-Gallego J, Nistal E, Jover R, Sánchez-Campos S.

20. **Spring Conference 2021: Gut microbiome & health. The Nutrition Society.** Online congress, 29/03/2021-30/03/2021. Poster presentation.
'A dietary intervention with Akkermansia muciniphila and quercetin supplementation reshapes gut microbiota composition in an in vivo model of early obesity related non-alcoholic fatty liver disease'. Juárez-Fernández M, Porras D, Petrov P, García-Mediavilla MV, Román-Sagüillo S, Martínez-Flórez S, González-Gallego J, Nistal E, Jover R, Sánchez-Campos S. *Proceedings of the Nutrition Society* 80 (OC2), E51. doi:10.1017/S002966512100063X.

21. Spring Conference 2021: Gut microbiome & health. The Nutrition Society. Online congress, 29/03/2021-30/03/2021. Poster presentation.
'Metagenomic and metabolomic shift on morbid obese patients undergoing bariatric surgery'. Román-Sagüillo S, García-Mediavilla MV, Juárez-Fernández M, Porras D, Linares P, Ballesteros-Pomar M, Urioste-Fondo A, Álvarez-Cuenllas B, González-Gallego J, Sánchez-Campos S, Jorquera F, Nistal E. *Proceedings of the Nutrition Society* 80 (OC2), E73. doi:10.1017/S0029665121000859.

22. XIV Jornadas de Investigadores del Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd). Barcelona (Spain), 9/11/2020 – 11/11/2020. Poster presentation.
'Combination of quercetin and Akkermansia muciniphila as an adjuvant therapy for early obesity and NAFLD through modulation of gut microbiota composition, lipid metabolism, bile acid pool and associated signalling pathways'. Juárez-Fernández M, Porras D, Nistal E, Petrov P, García-Mediavilla MV, Román-Sagüillo S, Martínez-Flórez S, Jorquera F, González-Gallego J, Jover R, Sánchez-Campos S.

23. XIV Jornadas de Investigadores del Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd). Barcelona (Spain), 9/11/2020 – 11/11/2020. Oral communication.
'MCJ-KO genotype determines a gut microbiota signature involved in a protective effect against non-alcoholic steatohepatitis'. Porras D*, Goikoetxea-Usandizaga N*, García-Mediavilla MV, Rodríguez H, Nistal E, Juárez-Fernández M, Martínez-Flórez S, Rincón M, Varela-Rey M, González-Gallego J, Abecia L, Anguita J, Martinez-Chantar M*, Sánchez-Campos S*.

24. XIV Jornadas de Investigadores del Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd). Barcelona (Spain), 9/11/2020 – 11/11/2020. *Poster presentation.*
'Gut microbiota composition and faecal metabolomic profile in morbid obese patients before and after undergoing bariatric surgery'. Román-Sagüillo S, Juárez-Fernández M, Porras D, García-Mediavilla MV, Linares P, Ballesteros-Pomar M, Urioste-Fondo A, Álvarez-Cuenllas B, González-Gallego J, Sánchez-Campos S, Jorquera F, Nistal E.

25. 1st International Conference World of Microbiome: Digestive & Metabolic Health. Vienna (Austria), 04/11/2020 – 06/11/2020. Poster presentation.
'Bariatric surgery modifies gut microbiota composition and associated metabolomic signature in obese patients'. Porras D, Juárez-Fernández M, García-Mediavilla MV, Román-Sagüillo S, Linares P, Ballesteros-Pomar M, Urioste-Fondo A, Álvarez-Cuenllas B, González-Gallego J, Sánchez-Campos S, Jorquera F, Nistal E.

26. 1st International Conference World of Microbiome: Digestive & Metabolic Health. Vienna (Austria), 04/11/2020 – 06/11/2020. Poster presentation.
'Dietary intervention together with Akkermansia muciniphila and quercetin administration restores intestinal dysbiosis in an in vivo model of early obesity and NAFLD'. Juárez-Fernández M, Porras D, García-Mediavilla MV, Martínez-Flórez S, Román Sagüillo S, Petrov P, Jover R, González-Gallego J, Nistal E, Sánchez-Campos S.

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'Transplantation of gut microbiota derived from MCJ-KO genotype determines a protective profile against non-alcoholic fatty liver disease in germ-free mice'. Porras D*, Goikoetxea-Usandizaga N*, García-Mediavilla MV, Rodríguez H, Nistal E, Juárez-Fernández M, Martínez-Flórez S, Bizkarguenaga M, Rincon M, González-Gallego J, Varela-Rey M, Abecia L, Anguita J, Martinez-Chantar M*, Sánchez-Campos S*. *Journal of Hepatology*. 73:S239. doi: 10.1016/S0168-8278(20)30981-8.

28. 45 Congreso Anual de la Asociación Española para el Estudio del Hígado (AEEH). Madrid (Spain), 12/02/2020 – 14/02/2020. Oral communication.
'Transferencia del efecto protector del genotipo MCJ-KO frente al desarrollo de la enfermedad de hígado graso no alcohólico a ratones libres de gérmenes mediante el trasplante de microbiota intestinal'. Porras D*, Goikoetxea-Usandizaga N*, García-Mediavilla MV, Rodríguez H, Nistal E, Juárez-Fernández M, Martínez-Flórez S, Rincon M, Varela-Rey M, González-Gallego J, Abecia L, Anguita J, Martinez-Chantar M*, Sánchez-Campos S*. *Gastroenterología y Hepatología*. 43: 23.

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'MCJ-KO genotype mediates protection against non-alcoholic fatty liver disease development in germ-free mice following intestinal microbiota transplantation'. García-Mediavilla MV*, Goikoetxea-Usandizaga N*, Porras D, Rodríguez H, Nistal E, Juárez-Fernández M, Martínez-Flórez S, Rincon M, Varela-Rey M, González-Gallego J, Abecia L, Anguita J, Martinez-Chantar M*, Sánchez-Campos S*.

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'Nutritional intervention with Akkermansia muciniphila and quercetin in the development of early obesity and non-alcoholic fatty liver disease (NAFLD)'. Nistal E, García-Mediavilla MV, Porras D, Juárez-Fernández M, Román-Sagüillo S, Petrov P, Martínez-Flórez S, Jorquera F, Jover R, González-Gallego J, Sánchez-Campos S.

31. I Reunión de Hepatología Traslacional (Asociación Española para el Estudio del Hígado, AEEH). San Sebastián (Spain), 04/10/2019 - 05/10/2019. *Poster presentation.*

'Efectos beneficiosos de la combinación de Akkermansia muciniphila y quercetina en un modelo in vivo de obesidad temprana y NAFLD'. Juárez-Fernández M, Porras D, García-Mediavilla MV, Martínez-Flórez S, Jorquera F, González-Gallego J, Nistal E, Sánchez-Campos S.

32. XIII Congreso Anual de Biotecnología (BAC). Madrid (Spain), 10/07/2019 - 12/07/2019. Poster presentation.

'Beneficial effects of a nutritional intervention with Akkermansia muciniphila and quercetin in a rat model of early obesity and NAFLD'. Juárez-Fernández M, Porras D, García-Mediavilla MV, Martínez-Flórez S, Jorquera F, González-Gallego J, Nistal E, Sánchez-Campos S.

Additionally, the spreading of the results obtained in these articles was performed not only by scientific congress, but also with the following contributions:

- **Short blog post on Encyclopedia Scholar Community.** Juárez-Fernández M, Román-Sagüillo S, Porras D, García-Mediavilla MV, Ballesteros-Pomar M, Alvarez-Cuenllas B, González-Gallego J, Sánchez-Campos S, Jorquera F, Nistal E. Long-Term Effects of Bariatric Surgery on Gut Microbiota. Encyclopedia. Available online: https://encyclopedia.pub/entry/15831.
- **Video abstract summarizing the article on Encyclopedia Scholar Community.** Juárez-Fernández M, Román-Sagüillo S, Porras D, García-Mediavilla MV, Linares P, Ballesteros-Pomar M, Fondo A, Alvarez-Cuenllas B, Sánchez-Campos S, Jorquera F, Nistal E, González-Gallego J. Bariatric Surgery and Gut Microbiota. Encyclopedia. Available online: https://encyclopedia.pub/video/video_detail/92.

- **Short blog post on Encyclopedia Scholar Community.** Juárez Fernández M, Porras D, Petrov P, Román-Sagüillo S, García-Mediavilla MV, Martínez Flórez S, González-Gallego J, Nistal E, Jover Atienza R, Sánchez-Campos S. Quercetin and *Akkermansia muciniphila*: Facing NAFLD & Obesity. Encyclopedia. Available online: https://encyclopedia.pub/entry/17375.
- **Video abstract summarizing the article on Encyclopedia Scholar Community.** Román-Sagüillo S, Juárez-Fernández M, Porras D, Petrov P, García-Mediavilla MV, Martínez-Flórez S, González-Gallego J, Nistal E, Jover Atienza R, Sánchez-Campos S. Childhood Obesity. Encyclopedia. Available online: https://encyclopedia.pub/video/video_detail/221.

During the whole predoctoral period, the following articles, which are not part of this PhD Thesis, have been also published:

» **Aging, gut microbiota and metabolic diseases: management through physical exercise and nutritional interventions.** Juárez-Fernández M, Porras D, García-Mediavilla MV, Román-Sagüillo S, González-Gallego J, Nistal E, Sánchez-Campos S. *Nutrients* (2021) 13(1): 16. doi: 10.3390/nu13010016.

» **Exercise training modulates the gut microbiota profile and impairs inflammatory signaling pathways in obese children.** Quiroga R, Nistal E, Estébanez B, Porras D, Juárez-Fernández M, Martínez-Flórez S, García-Mediavilla MV, de Paz JA, González-Gallego J, Sánchez-Campos S, Cuevas MJ. *Experimental & Molecular Medicine* (2020) 52(7):1048-1061. doi: 10.1038/s12276-020-0459-0.

» **Beneficial effects of exercise on gut microbiota functionality and barrier integrity, and gut-liver crosstalk in an *in vivo* model of early obesity and non-alcoholic fatty liver disease.** Carbajo-Pescador S, Porras D, García-Mediavilla MV, Martínez-Flórez S, Juárez-Fernández M, Cuevas MJ, Mauriz JL, González-Gallego J, Nistal E, Sánchez-Campos S. *Diseases Models & Mechanisms* (2019) 12(5): dmm039206. doi: 10.1242/dmm.039206.

LIST OF ABBREVIATIONS

AASLD	American Association for the Study of Liver Diseases
ACC	Acetyl-CoA carboxylase
ALT	Alanine aminotransferase
ASMBS	American Society for Metabolic and Bariatric Surgery
AST	Aspartate aminotransferase
ATF6	Activating Transcription Factor 6
ATP	Adenosine triphosphate
BA	Bile acid
BAAT	Bile acid-CoA: amino acid N-acyltransferase
BiP	Binding immunoglobulin protein
BMI	Body mass index
BPD/DS	Bilipancreatic diversion with duodenal switch
BSEP	Bile salt export pump
BSH	Bile salt hydrolase
CA	Cholic acid
CAP	Controlled attenuation parameter
CCL2	C-C motif chemokine ligand 2
CCR	C-C motif chemokine receptors
CDCA	Chenodeoxycholic acid
CFU	Colony-forming unit
ChREBP	Carbohydrate response element-binding protein
CNS	Central nervous system
COVID-19	Coronavirus disease
COX2	Cyclooxygenase-2
CVD	Cardiovascular disease
CXCL10	C-X-C motif chemokine ligand 10
CYP27A1	Sterol 27-hydroxylase
CYP7A1	Cholesterol 7α-hydroxylase
CYP8B1	Cholesterol 8β-hydroxylase
DAG	Diacylglycerol
DAMP	Damage-associated molecular pattern
DCA	Deoxycholic acid

DEXA	Dual X-ray absorptiometry
DGAT2	Diacylglycerol O-acyltransferase 2
DNL	*De novo* lipogenesis
EASL	European Association for the Study of the Liver
EMA	European Medicines Agency
ENS	Enteric nervous system
ER	Endoplasmic resticulum
ETC	Electron transport chain
F:B ratio	Firmicutes/Bacteroidetes ratio
FAO	Fatty acid oxidation
FAS	Fatty acid synthase
FATP	Fatty acid transporter protein
FDA	Food and Drug Administration
FFA	Free-fatty acid
FFAR	Free fatty acid receptor
FGF	Fibroblast growth factor
FLI	Fatty liver index
FMT	Faecal microbiota transplantation
FXR	Farnesoid X receptor
GABA	Gamma-aminobutyric acid
GPBAR1	G protein-coupled bile acid receptor 1
GF	Germ-free
GLP	Glucagon-like peptide
GPR	G-protein-coupled receptor
GRP	Glucose regulated protein
GVB	Gut vascular barrier
GWAS	Genome-wide associated analysis
HBV	Hepatitis B virus
HCC	Hepatocarcinoma
HCV	Hepatitis C virus
HDCA	Hyodeoxycholic acid
HDL	High-density lipoprotein
HFD	High-fat diet
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance

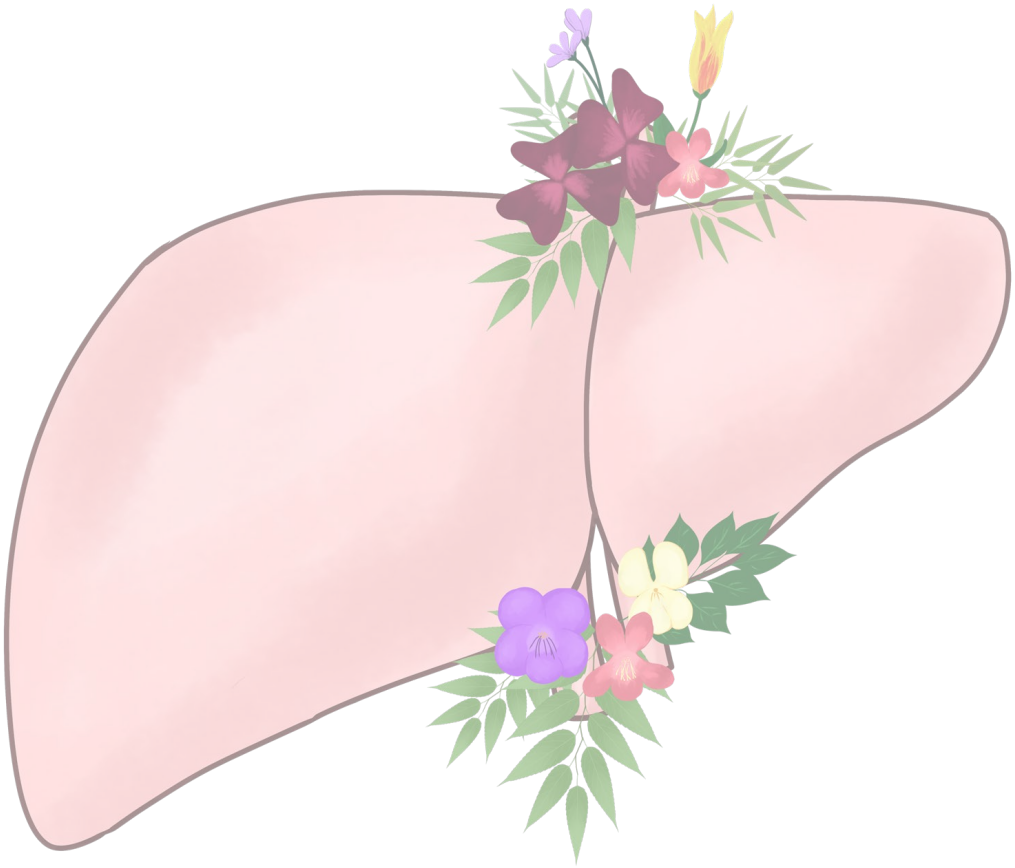
HSC	Hepatic stellate cells
HSI	Hepatic steatosis index
IGF	Insulin growth factor
IL	Interleukins
IR	Insulin resistance
IRE1	Inositol-Requiring Enzyme 1
IRS	Insulin receptor substrate
JAM	Junctional adhesion molecule
JNK	c-Jun N terminal kinase
LBP	Lipopolysaccaride binding protein
LCA	Lithocholic acid
LPS	Lipopolysaccaride
LXR	Liver X receptor
MAFLD	Metabolic-associated fatty liver disease
MCA	Muricholic acid
MCJ	Methylation-controlled J protein
MDC	Murideoxycholic acid
MDR	Multidrug resistance protein
MMP	Metallopeptidase
MONW	Metabolically obese normal weight
MRI	Magnetic resonance imaging
MRI-PDFF	Magnetic resonance imaging-derived proton density fat fraction
MRP2	Multidrug resistance-associated protein 2
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
NAD	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
NAMPT	Nicotinamide phosphoribosyltransferase
NAS	NAFLD Activity Score
NASH	Non-alcoholic steatohepatitis
NF-κB	Nuclear factor κB
NLRP3	NLR family pyrin domain containing 3
NOD	Nucleotide oligomerization domain
NTCP	Sodium taurocholate cotransporting polypeptide

OATP	Organic anion transporting polypeptide
OXPHOS	Oxidative phosphorylation
PAMP	Pathogen-associated molecular pattern
PERK	Protein kinase RNA-Like ER kinase
PGC	Peroxisome proliferator-activated receptor-gamma coactivator
PI3K	Phosphatidylinositol 3-kinase
Plin2	Perilipin 2
PNPLA3	Patatin like phospholipase domain containing 3
PPAR	Peroxisome proliferator-activated receptor
PPAR-α	Peroxisome proliferator-activated receptor-α
PRR	Pattern recognition receptor
PXR	Pregnane X receptor
PYY	Peptide YY
ROS	Reactive oxygen species
RYGB	Roux-en-Y gastric bypass
SAT	Subcutaneous adipose tissue
SCD-1	Stearoyl CoA-desaturase-1
SCFA	Short-chain fatty acid
SHP	Small heterodimer partner
SIBO	Small intestinal bacterial overgrowth
SIRT	Sirtuins
SOD	Superoxide dismutase
SR	Sustained release
SREBP	Sterol regulatory element-binding protein
T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerol
TCA	Tricarboxylic acid cycle
TGF- β	Transforming growth factor-β
TGR5	Takeda G protein-coupled receptor 5
THR-β	Thyroid hormone receptor β
TJ	Tight junction
TLR	Toll-like receptor
TMA	Trimethylamine
TMAO	Trimethylamine N-oxide

TNF	Tumor necrosis factor
UDCA	Ursodeoxycholic acid
UPR	Unfolded protein response
VAT	Visceral adipose tissue
VLDL	Very-low density lipoproteins
VSG	Vertical Sleeve gastrectomy
WD	Western diet
WHO	World Health Organization
ZO	Zonula occludens
α-SMA	α-smooth muscle actin

LIST OF FIGURES

LIST OF TABLES



STATE OF THE ART

1. OBESITY

1.1. INCIDENCE, PREVALENCE AND DEFINITION

Obesity was firstly recognised as a disease in 1997 by the World Organization of Health (WHO) and consolidated one year later with the evidence published by the National Institutes of Health[1,2]. Nowadays, twenty-five years later, obesity has become a pandemic and is considered one of the main public matters in the world. In fact, the WHO, in its European Regional Obesity Report of 2022, states that overweight and obesity have a prevalence of 60% in adults and nearly 30% in children[3]. Additionally, while the prevalence of obesity in United States is approximately 42.4%, in Europe the average stands at 24%[4]. In our country, the data is still worrying: in 2016, the 23.8% of Spanish adults had obesity[3], and, in 2019, the 17.3% of kids between 6 and 9 years-old had obesity and the 23.3% had overweight[5].

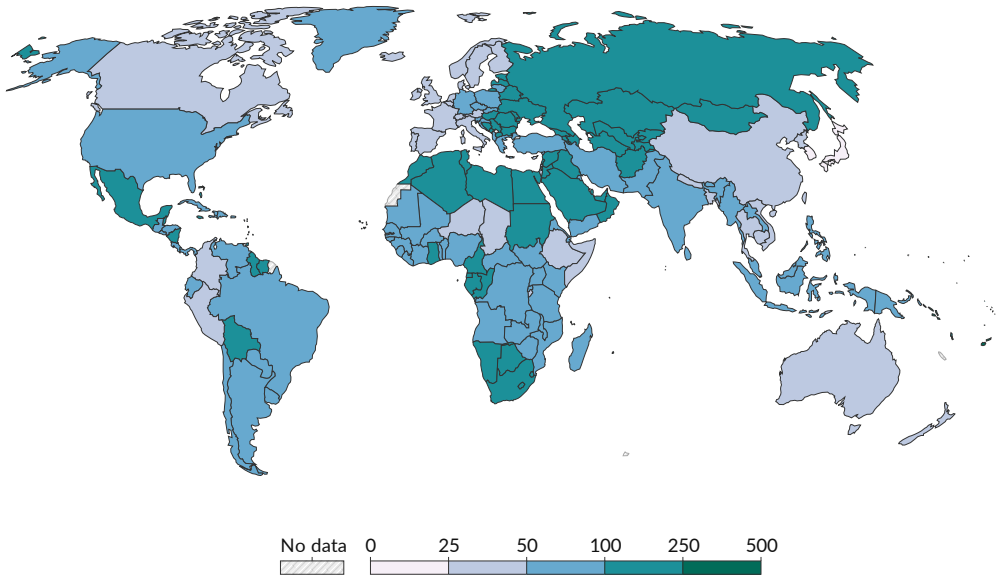


Figure 1. Estimated annual number of deaths attributed to obesity per 100.000 people in 2019. Obtained from Our World In Data (OurWorldInData.org/obesity • CC BY Source: IHME, Global Burden of Disease).

Moreover, this disease is responsible of a 5% of mortality in the world[6], with more than 4 million of deaths per year (**Figure 1**). In fact, it is expected that adults with overweight and with an age between 20 and 29 years will lose 3.3 years of life, while those with obesity will experiment a decrease of 5.6 to 10.3 years in their life expectancy due to the disease[7]. In this sense, it is estimated that every increase of 5 kg/m^2 above the

upper limit of normal body weight (25 kg/m^2) raises the overall mortality by 30%[8]. Accordingly, the burden of the public health care systems and the consequent worldwide economies are enormously compromised[9]. In fact, between 0.7% and 2.8% of the costs of the health care systems of a country are being destined for obesity-related issues[10]. In addition, the increasing trends point out that obesity will become worse, with an incidence of one in two adults with obesity and one in four adults with severe obesity in 2030[11].

But… This is not only a matter of numbers, so the question that grasps our minds is: '*What is obesity?*' Obesity is a multifactorial disease defined as the excessive accumulation of adipose mass in the body with a low-grade chronic inflammation[3]. Additionally, fat accumulation is commonly related to the appearance of hypertension, hyperinsulinemia, dyslipidaemia, hypercholesterolemia, or impaired fasting glucose, between others[12]. Obesity can be measured using the body mass index (BMI), calculated as the body weight (expressed in kilograms) divided by the height squared (expressed in square meters)[13]. The WHO establishes the classification of nutritional status based on BMI measurement as shown in Table 1.

Table 1. Classification of the nutritional status based on the body mass index.

BMI (kg/m^2)	Nutritional status
Below 18.5	Underweight
18.5 – 24.9	Normal weight
25.0 – 29.9	Overweight
30.0 – 34.9	Obesity class I
35.0 – 39.9	Obesity class II
Above 40	Obesity class III

BMI: body mass index.

The importance of obesity does not only fall on the disease itself, but also on the associated comorbidities that have been identified, such as cardiovascular disease (CVD), diabetes, chronic kidney disease, obstructive sleep apnea, degenerative joint disease, liver pathologies like non-alcoholic fatty liver disease (NAFLD), and cancer[4,14,15]. In fact, the susceptibility of patients with obesity to other health problems was highlighted during the pandemic of coronavirus disease (COVID-19), in which people with obesity that were infected with the virus showed a higher risk of death[16].

1.2. PATHOGENESIS

Once the worldwide relevance of obesity has been clarified, it is important to deepen in the mechanisms and factors that provoke its appearance and development. In general terms, obesity constitutes the consequence of a long-term imbalance between the energy intake and the energy expenditure[17]. However, if it were that simple, this pandemic would not have become a severe problem. In the pathogenesis of obesity, it is not only a matter of calories, since plenty of factors such as genetics, epigenetics, socioeconomic status, or cultural lifestyle influence on how each individual behaves with food[18]. Therefore, not only hormonal, nutritional, and metabolic factors but also psychological, environmental and social ones are involved in obesity development, as well as their complex interrelationships, which leads to a framework difficult to totally comprehend[19] (**Figure 2**).

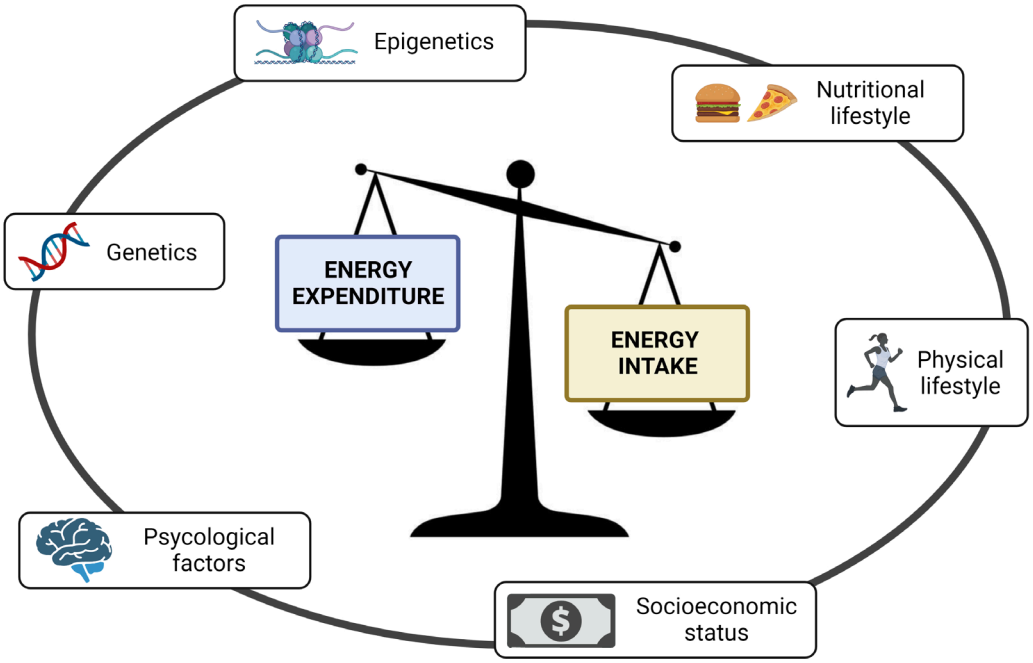


Figure 2. Etiology of obesity. A disbalance between the energy intake and the energy expenditure, together with the influence of different factors such as genetics, epigenetics, psychological status, socioeconomic status, or nutritional and physical lifestyle constitute the main causes of obesity.

Regarding the role of genetics and epigenetics in the pathogenesis of this disease, BMI heritability has been established between 40% and 70%, and is a factor that undoubtedly influences on the response that each individual has to the obesogenic environment[20]. Moreover, genome-wide associated analysis (GWAS) has identified

many loci in which sequence variations are strongly associated with BMI, although they could only explain a difference of 3-5% in this parameter[21]. In this sense, other factors such as age, sex, neuroendocrine aspects, socio-cultural and geographical status, concomitant medications, and mental health could affect this positive energy balance and contribute to obesity development[4].

Based on the cause of the disease, it can be distinguished between monogenic obesity, a rare form of the disease in which the mutation of specific genes related to body weight regulation is the key developmental factor, and primary obesity, in which different polymorphisms can be predisposing to the disease but are not the main cause of it[9]. Furthermore, metabolically obese normal weight (MONW) refers to a type of obesity in which the patients have normal BMI but present metabolic alterations, such as hypertension, insulin resistance, type 2 diabetes mellitus (T2DM), and hypertriglyceridemia. MONW appears due to the excess of deposition of visceral adipose, much more hormonally and metabolically active compared to other types of adipose tissue[11].

As obesity is defined as an accumulation of adipose mass, in order to comprehend its pathogenesis, we will first focus on this tissue. Firstly, the excess of energy derived from food is stored as triacylglycerols (TAGs) in the subcutaneous adipose tissue (SAT) through the proliferation (hyperplasia) and the increase in size (hypertrophy) of the adipocytes[22]. However, in obesity, the long-term excess of energy provokes that subcutaneous adipocytes reach their own limit in their hypertrophy and hyperplasia ability, fact that leads to a cellular stress. This stress is related to a status of hypoxia and an induction of oxidative stress, mechanical stress, presence of toxic metabolites, inflammation, and cell death, which finally provokes adipocyte dysfunction[23]. Furthermore, reaching the limit of energy storage by the SAT also causes an increase in the lipolytic activity of the adipocytes, which leads to the hydrolysis of TAGs and the consequent release of free-fatty acids (FFAs) into the systemic circulation. These FFAs are ectopically accumulated in other organs such as skeletal muscle, liver, or visceral adipose tissue (VAT), producing in them lipotoxicity, dyslipidaemia, insulin resistance and inflammation[23] (**Figure 3**). The basis to understand the lipotoxicity of the ectopic fat accumulation resides in the fact that FFAs are the substrate for the synthesis of ceramides, compounds able to contribute to insulin resistance. Moreover, the adipocytes' stress, as well as the accumulation of TAGs in VAT and other organs, induce an inflammatory

response that affects metabolic functions and, specially, impairs insulin sensitivity. During this response, immune cells infiltrate the adipose tissue, and favour the secretion of proinflammatory mediators such as tumour necrosis factor (TNF) or interleukins that also contribute to the inflammatory status[22].

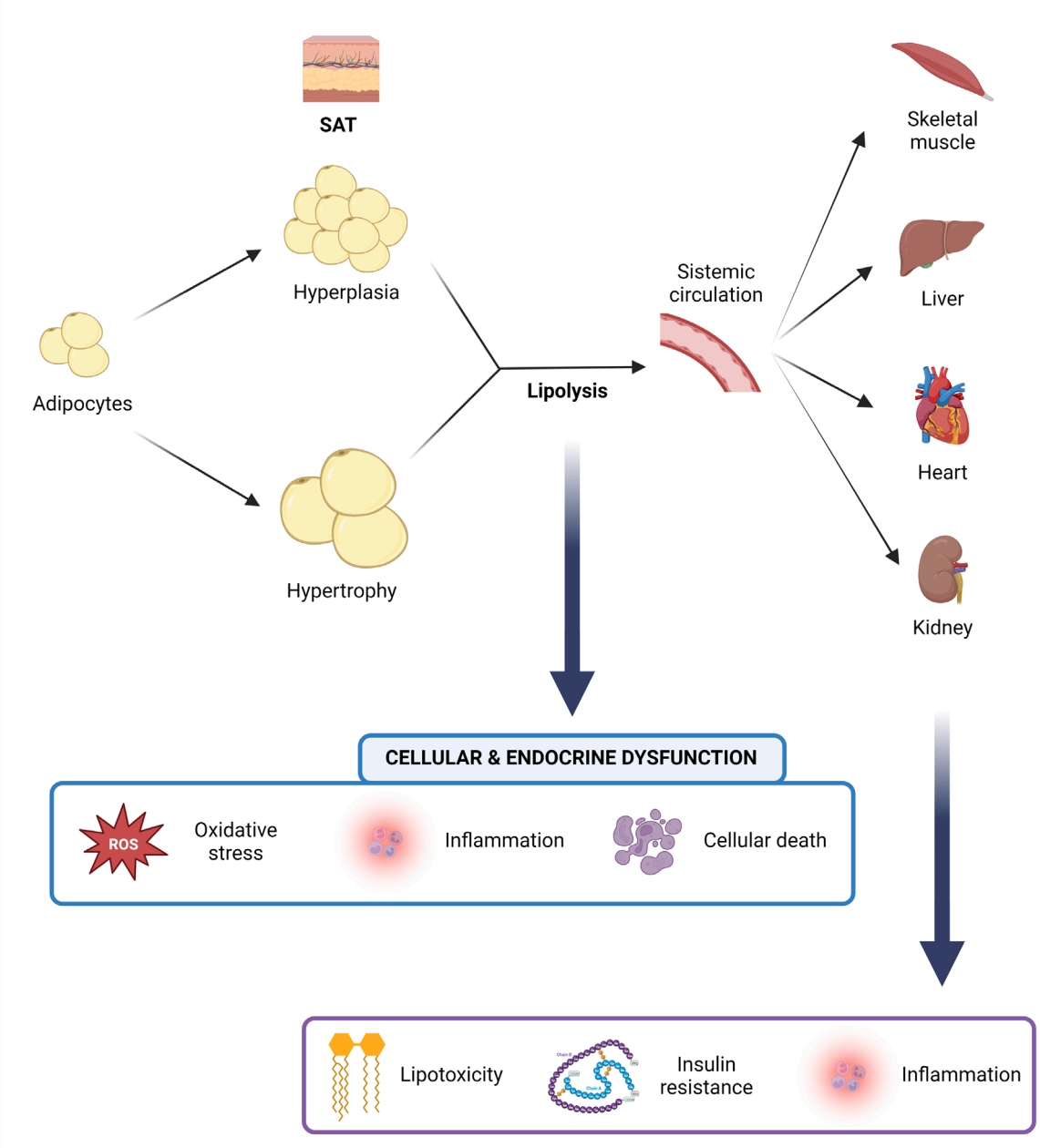


Figure 3. Pathogenesis of obesity. During a prolonged period of high energy intake, the adipocytes reach their limit of hyperplasia and hypertrophy, leading to the activation of lipolysis process and the consequent release of free-fatty acids to the systemic circulation and the cellular and endocrine dysfunction. The lipids are accumulated in different organs such as skeletal muscle, liver, heart, or kidney, promoting lipotoxicity, insulin resistance and inflammation, whereas the adipose dysfunction also leads to oxidative stress, inflammation and cellular death. SAT, subcutaneous adipose tissue; ROS, reactive oxygen species.

Furthermore, the dysfunction of the adipocytes and the local inflammation are associated with an alteration of the endocrinal function of the tissue. In a healthy status, SAT acts as an endocrine organ that secretes different hormones, cytokines and proteins called adipokines[17]. Among them, the role of leptin and adiponectin in the regulation of satiety and appetite has been deeply investigated. Leptin is a hormone secreted in response to a meal, whose main function in the hypothalamus is the suppression of appetite and food intake. Moreover, this hormone is also involved in the regulation of glucose and lipid metabolism: it increases glucose uptake in the liver and supresses this process in adipose tissue; additionally, leptin also favours fatty acid oxidation in both liver and adipose tissue[24]. But… *what happens with this hormone during obesity?* As leptin levels are proportional to BMI, patients with obesity have a high concentration of this hormone. However, the main target tissues such as liver, skeletal muscle, adipose tissue, or brain are resistant to its effects, possibly due to the associated inflammatory and oxidative status[23]. These high concentrations of leptin also contribute to the inflammatory status. On the other hand, adiponectin is a hormone related to a starvation signal, able to inhibit the accumulation of FFAs and to maintain the glucose homeostasis[25]. Thus, adiponectin promotes the capacity of hyperplasia of the adipocytes, as well as potentiates the glucose intake in muscle, adipose tissue, and liver, and decreases the metabolization of lipids. Nevertheless, adiponectin levels are decreased in obesity[23].

Altogether, a straightforward explanation of the complex process that is behind the obesity development is brought up, in which a plenty of diverse factors, mentioned above, play an important role. Therefore, an early diagnosis as well as an effective treatment is urgently needed to face this pandemic.

1.3. Diagnosis and Treatments

The initial diagnosis of obesity is currently based on the BMI, a useful, standardized, fast and simple tool with a low associated cost[9]. However, the BMI as a diagnostic method has some limitations: the impossibility to distinguish between fat and lean mass, as well as its inability to quantify adiposity and to indicate body fat distribution. In fact, the classification of obesity based on BMI can be inaccurate for people with a high proportion of muscle or bone mass, or for people with an excessive and abnormal body fat accumulation[9,26]. Additionally, using this anthropometric measurement indistinctively for all the ages and ethnicities is unprecise. In this sense, although age-

related cut-offs have been applied, specifically for childhood, there is no consensus about the appropriate limits. Moreover, regarding ethnicity, even though obesity and overweight cut-offs for Asian people have been suggested to be adjusted, they have not been included in the WHO guidelines[3,14].

For the above-mentioned reasons, the BMI is an approximate method, and it should be complemented with other anthropometric or image-based measurements. Waist-to-hip ratio, waist-to-height ratio, waist circumference, neck circumference or skinfold-based technique are other anthropometric measurements able to determine the fat distribution[3,26]. These strategies are cheap and easily performed but, similar to BMI, they also have drawbacks mainly due to the error made by the operator, which ranges from 3 to 4%. Therefore, the image tools such as computed tomography, nuclear magnetic resonance, or densitometry techniques like dual X-ray absorptiometry (DEXA) are the most accurate techniques, highlighting their precision and invasiveness, quality-price ratio, and time as the main advantages[14,26]. However, these measurements are more difficult to assess and need specific and additional computational process[9].

In the last years, with the development of high throughput methods, the search for different metabolic, epigenetic, or microbial biomarkers that could make the diagnosis of obesity more accurate and refined has drastically increased. The insulin/insulin-like growth factor (IGF) axis, different adipokines such as leptin or the chronic low-grade inflammation pathway have been suggested as a possible niche of biomarkers. Nevertheless, more studies are needed to further understand these links before its application in the clinical practice[14].

Once the diagnosis of obesity has been clarified, it is necessary to delve into the available therapeutic options, which are chosen based on the characteristics of each patient. Therefore, in general terms, the strategies to treat obesity can be classified into non-surgical and surgical treatments (**Figure 4**).

1.3.1. Non-surgical treatments

The intricacy of obesity requires an individualized therapy, as well as the patient predisposition and motivation, which is essential to obtain positive results. Dietary intervention and exercise practice constitute the ideal approach to maintain body weight

and is the common choice for those patients with a BMI between 25 kg/m^2 and 26.9 kg/m^2 and the basis for patients with a BMI greater than 27 kg/m^2 [13].

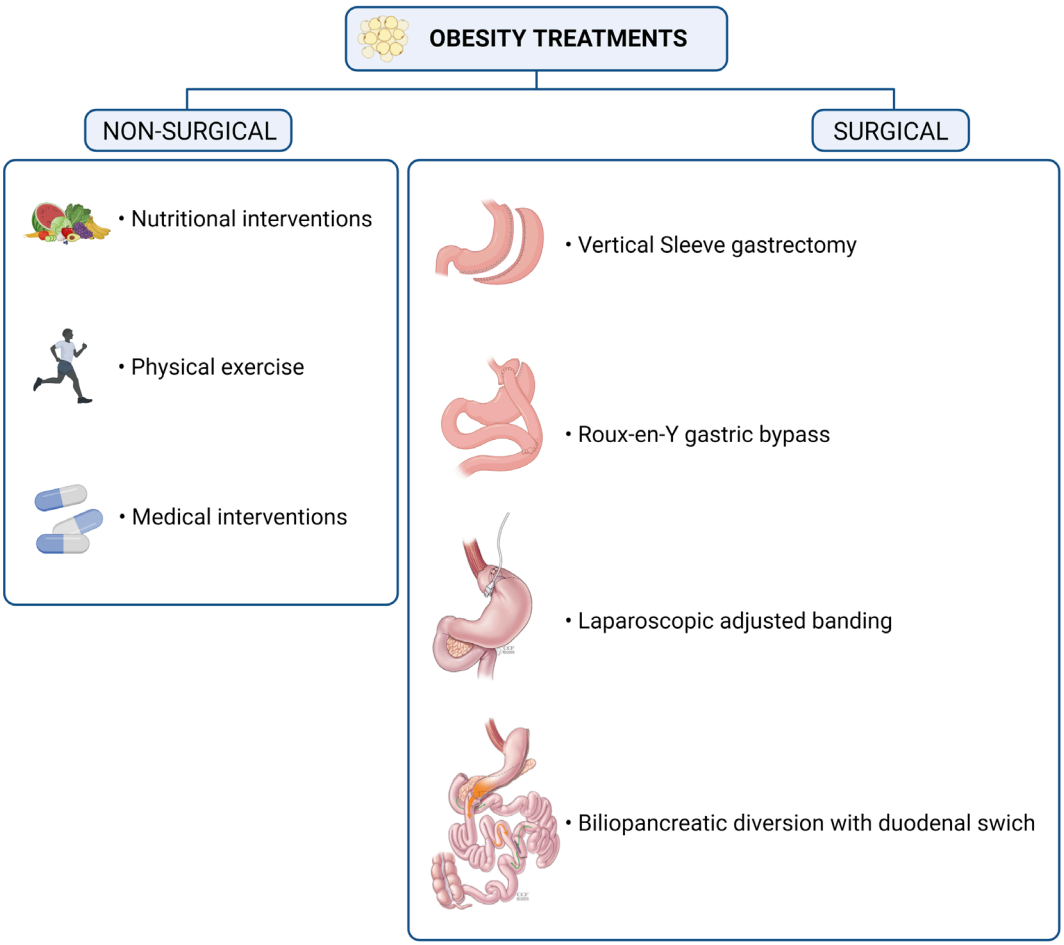


Figure 4. Available treatments for obesity. The main therapeutic strategies to deal with obesity are classified into non-surgical (nutritional interventions, physical exercise, and drugs), and surgical treatments, commonly known as bariatric surgery (mainly, vertical sleeve gastrectomy, Roux-en-Y gastric bypass, laparoscopic adjusted banding, and biliopancreatic diversion with duodenal switch).

Regarding nutritional interventions, there are many different options that could be selected based on the characteristics of the patient: energy-restricted diets, balanced-nutrient low-energy diets, portion-controlled diets, intermittent energy restriction plans, low-fat diets, etc. In this sense, although caloric restriction is the main determinant of weight loss, the most important factor is patients' adherence to a diet able to produce a deficit of energy and, consequently, a maintained weight loss[27]. Furthermore, physical activity is, together with dietary intervention, the first approach to face obesity. Different exercise programmes have demonstrated benefits in body weight loss and in disease-

related status, although there is still no consensus about the optimal dose or type of exercise[28].

However, in many cases, nutritional intervention combined with physical exercise is not enough, so it is recommended to include pharmacological treatment or even consider surgical approaches. But the question is… *When should the pharmacological treatment be implemented?* The selection of the best treatment for each patient is based on the BMI and on the presence of comorbidities[13]. The guidelines establish the pharmacological treatment as the main strategy in patients with a BMI higher than 30 kg/m^2 and patients with BMI higher than 27kg/m^2 with one or more comorbidities, being necessary to evaluate the efficacy of the treatment after the first three months[4]. If this approach is not effective and the patient does not achieve a weight loss of 5% after 3 months, the treatment should be suspended[13]. Thus, the main aim of medical treatments is to maintain weight loss at long term, and consequently, let the patient improves not only the comorbidities but also their quality of life and life expectancy. Currently, the Food and Drug Administration (FDA) approved drugs for treating obesity at long-term are orlistat, phentermine, phentermine/topiramate extended release, naltrexone sustained release (SR)/bupropion SR, liraglutide and semaglutide, while the European Medicine Agency (EMA) have approved the same drugs, except phentermine/topiramate extended release, and semaglutide[4]. However, other medications such as diethylpropion, phendimetrazine, benzphetamine, and phentermine have been approved by FDA and EMA for short-term obesity treatment in patients with a BMI equal or higher than 30 kg/m^2 that have not previously responded to other strategies[4]. In Spain, the recommended drug is liraglutide, and if it is not effective or is contraindicated, orlistat or the combination of naltrexone, bupropion and norepinephrine extended release constitute the other options[13]. Additionally, metformin and inhibitors of the sodium-glucose cotransporter-2 are also used in the clinical practice[7]. Despite the existence of different alternatives, medical treatments only reach a weight loss of 3% to 10% of total body weight, and, in many cases, they do not succeed at long term[7].

To sum up, even though diet and exercise are the safest approaches to face obesity, they are effective at long term only in a tiny percentage of patients. Moreover, the available medical treatments for obesity are common to fail and not to reach the desirable weight loss. For those reasons, there is an urgent need for new research and development of novel alternatives to efficiently treat obesity[16].

1.3.2. Surgical treatments

Surgical treatment, commonly known as bariatric surgery, is the carried approach for patients with severe obesity. In fact, surgical treatment in adults between 18 and 65 years old is indicated in patients with a BMI higher than 40 kg/m^2, in patients with a BMI between 35 kg/m^2 and 39.9 kg/m^2 with at least one related comorbidity or in patients with a BMI between 30 kg/m^2 and 34.9 kg/m^2, related comorbidities and who have not lost significant body weight with non-surgical strategies. In the case of adolescents or adults older than 65 years, an individualized study to evaluate the safest and most effective approach is previously needed[13,29].

Bariatric surgery procedure involves a complete multidisciplinary team and many different steps that begin with the pre-operative: patient needs to satisfy the aforementioned inclusion criteria of BMI and comorbidities, as well as to undergo a psychological evaluation, a nutritional education plan and an assessment of medical risks[30]. In this sense, a preoperative weight loss of 5-10% is recommended due to its intraoperative advantages, the reduction of the hospital stay period and the increase in the adherence of life changes[13]. Moreover, after the surgical procedure, the patient should follow medical instructions, such as the nutritional plan and lifestyle change, as well as endure a medical follow-up that usually occurs during the two-month to two-year postoperative period[30].

The available surgical procedures are classified into three different groups based on the mechanism of action: volume restrictive, nutrient malabsorptive, or its combination. Moreover, depending on the route of access, the techniques can also be classified as endoscopic or laparoscopic. Thus, volume restrictive techniques reduce the gastric capacity and result in a limitation of the caloric intake due to the early feeling of satiety, whereas malabsorptive techniques decrease the length of the small intestine, reducing as a consequence the efficacy of nutrient absorption. Despite the profound weight loss that malabsorptive techniques achieve, the complications of these procedures due to the consequences of malabsorption have led to substitute them for a combination of restrictive and malabsorptive procedures[15,30].

The four more important surgical procedures that are being currently performed in the clinical practice[29] are summarized in **Figure 4** and explained as follow:

- **Vertical Sleeve gastrectomy (VSG).** This volume-restrictive surgical technique consists in removing approximately the 80% of the stomach using surgical staplers[29]. Currently, it is one of the most popular procedures for the treatment of obesity worldwide because of its simplicity and advantages[30]. Regarding its commonest complications, it could be mentioned the development or worsening of gastroesophageal efflux disease, gastric fistulas, weight regain, or gastric stenosis, among others[13,15].

- **Roux-en-Y gastric bypass (RYGB).** This surgery, together with VSG, is one of the most common procedures and is based on a combination of restrictive and malabsorptive techniques[13]. Therefore, RYGB consists in dividing the stomach into a small top portion, called the pouch, and a larger portion that is bypassed, so it is not able to digest food. Additionally, the small intestine is connected to the pouch and the portion of the small intestine that was connected to the larger portion of the stomach is reconnected to the small intestine, resembling the shape of letter Y[29]. The main complications are postoperative hemorrhages, intestinal occlusion, ulcers, or nutritional complications[13]. In this sense, it is worth mentioning that some studies suggest that this procedure induces more weight loss than VSG[31].

- **Laparoscopic adjusted banding.** Surgery recommendable for women with BMI under 50 kg/m^2 [13] which consists in a silicone device that is placed around the stomach in its top part, reducing the amount of food intake. Currently, it is not usually performed due to its mechanical complications, the high risk of revision, removal or reoperation of the band and the suboptimal weight loss[29,32].

- **Bilipancreatic diversion with duodenal switch (BPD/DS).** BPD/DS consists in modifying the volume of the stomach in order to create a pouch with a sleeve-like shape and then to detach the first portion of the small intestine from the stomach. Next, a segment of the small intestine is connected to the outlet of the created stomach, so the food goes through the sleeve-like stomach and directly goes to the latter part of the intestine[29]. This type of surgery is associated with the greatest weight loss, but also with the highest ratio of complications, such as long-term diarrhea, abdominal bloating, or lower levels of fat-soluble vitamins, due to its malabsorptive component[15].

In addition, endoscopic techniques can be used for the treatment of primary obesity based on a previous individualized evaluation[7]. Herein, the main alternatives are:

- Aspiration therapy, which actively aspirates the ingested food located in the stomach by a percutaneous gastrostomy, reducing as consequence the caloric intake[33].
- Endoscopic gastroplasty, which modifies the physiology and anatomy of the stomach due to the plication of the gastric walls and the consequent reduction of the intragastric volume by up to 75%[13,33].
- Intragastric balloon. The restriction of the intake occurs due to the placement of a temporal balloon in the gastric lumen[33].

Although bariatric surgery is the most effective available treatment for inducing weight loss in patients with obesity[31], approximately 20-30% of patients do not lose enough weight[4] and 20-25% of patients regain some weight after the procedure[34], being necessary to also implement pharmacological treatment. Different reasons such as the surgical procedure, genetic polymorphisms, social and demographical factors, or behavioral, psychological, and biological mechanisms could be partially responsible of body weight regain or insufficient body weight loss[8,34]. This matter, together with the intrinsic risks of bariatric surgery and the limited efficiency of the medical treatments, reflect the real need of new alternative strategies to face obesity. Additionally, considering that obesity is a multifactorial pathology, the combination of different therapeutic approaches instead of a single treatment could be the key to achieve not only the desirable body weight loss, but also its maintenance at long term[4].

2. NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

2.1. INCIDENCE, PREVALENCE AND DEFINITION

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease in the world[35]. In fact, this pathology is nowadays the unique chronic liver disease with a rising tendency, while others such as hepatitis B virus (HBV) or hepatitis C virus (HCV) remain flat or even decreasing[36]. NAFLD includes a broad spectrum of diseases characterized by the accumulation of fat in the hepatocytes non-related to alcohol consumption, viral infection, medication, or other cause of liver disease[37]. It begins with

a benign condition called simple steatosis, defined as the accumulation of triacylglycerol (TAG) in more than 5% of the hepatocytes. This simple steatosis can progress to steatohepatitis (NASH), a pathological condition characterized by the presence of not only fat accumulation but also inflammation and hepatocyte damage[38]. NASH is a dynamic condition, being able to revert to simple steatosis, remain stable or further progress to fibrosis, cirrhosis or even hepatocellular carcinoma (HCC)[37]. In this sense, it has been estimated that NAFLD progresses to NASH in 30% of the cases, whereas 20% of patients with NAFLD show regression[39]. Furthermore, between 15% and 20% of NASH patients progress to cirrhosis, although these data are underestimated due to the huge prevalence of NAFLD and the existent underdiagnosis[40] (**Figure 5**).

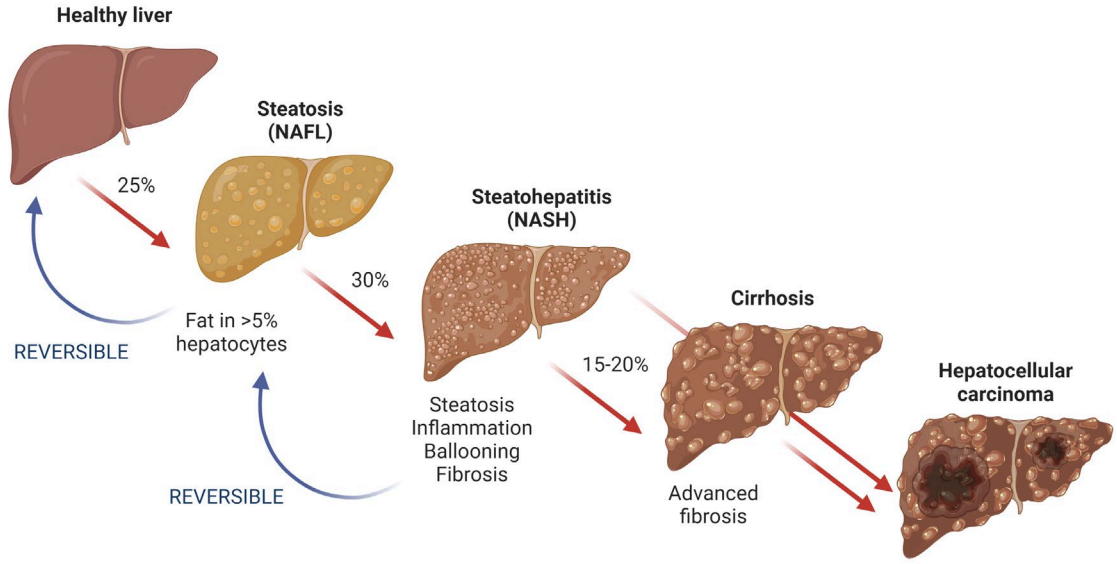


Figure 5. Non-alcoholic fatty liver disease: spectrum and progression. Approximately, 25% of the worldwide population develops steatosis, characterized by the accumulation of fat in more than 5% of the hepatocytes. This benign condition progresses in 30% of the cases to steatohepatitis, characterized not only by steatosis, but also by inflammation, ballooning, and fibrosis. Both steatosis and steatohepatitis are reversible conditions. 15-20% of NASH patients progress to a more complicated status, mainly cirrhosis and hepatocellular carcinoma.

Regarding its prevalence, one out of four people in the world has NAFLD and between 3% and 5% of worldwide population has NASH[39,41]. Additionally, it is estimated that the prevalence of NAFLD will reach the percentage of 56% between 2019 and 2030[42]. However, these data change geographically: whereas Africa has the lowest prevalence of NAFLD in the world with a 13%, South America and Middle East are the regions with the highest numbers (31% and 32%, respectively). Furthermore, the prevalence in US and Europe is similar to the worldwide data, and, in Asia, 27% of population presents the

disease[43,44]. NAFLD development is also a concern in the child population. Based on the aforementioned shocking numbers of children with obesity and considering that weight gain during the childhood and adolescence is able to potentially increase the susceptibility to NAFLD development during the adulthood[43], the future of NAFLD is alarming. In fact, a meta-analysis evaluated the prevalence of pediatric NAFLD between 2000 and 2021 and concluded that, while the 7.40% of the general child population has NAFLD, this prevalence increases to 52.49% in children with obesity[45].

The prevalence of NAFLD not only varies due to the geographical area, since there are other factors such as age, sex, genetic predisposition, and ethnicity that have a strong influence in the disease progression[39]. Briefly, as age increases, the prevalence of NAFLD rises. Regarding the ethnicity, Hispanics are the ones with the highest prevalence, whereas African Americans have the lowest value[36]. Moreover, NAFLD incidence is also different among sexes: while during reproductive age, the prevalence of the disease is higher in men than in women, after menopause, these data are equated[46]. Lastly, genetic predisposition is also a contributor factor to NAFLD development, as different polymorphisms have been related to the disease, such as the single nucleotide polymorphism present in the patatin-like phospholipase domain containing 3 (PNPLA3) gene, which has been associated with hepatic lipid accumulation; or the C-482T and T-455C polymorphisms in the APOC gene, which have been related to insulin resistance[36].

Similar to obesity, NAFLD spectrum is associated not only with a huge economic burden, but also with a decrease in the health-quality of life and a high risk of mortality, with cardiovascular diseases as the main leading cause of death[39,47,48]. Furthermore, the presence of liver fibrosis in NAFLD patients has been identified as the main factor related to a significant increase in mortality[35,49].

The importance of NAFLD cannot be explained without highlighting its well-established association with other pathologies such as obesity, T2DM and metabolic syndrome[38,43]. In fact, NAFLD is considered the hepatic manifestation of metabolic syndrome[47], a condition defined by the presence of at least three of the following alterations: increased triglyceride levels, reduced levels of high-density lipoprotein (HDL) cholesterol, abdominal obesity, hyperglycemia, and increased blood pressure[50]. Likewise, the relationship between NAFLD and obesity is very narrow: while the worldwide prevalence of NAFLD in general population is approximately 25%, it increases to nearly 90% regarding people with obesity[43]. In this sense, the tendency in the prevalence of

NAFLD shows the same increasing pattern than obesity[48]. Even though this strong link with obesity, NAFLD also takes place in people with normal weight, called lean NAFLD. Lean NAFLD is the term that refers to the presence of NAFLD without obesity, and the main associated factors are high amounts of visceral adipose tissue, high intake of fructose and fat, as well as genetic factors[43]. The global prevalence of non-obese NAFLD is 12.1%[51], being especially common in Asian population[47] and it is worthy to mention that a large percentage of people with lean NAFLD are also diagnosed as MONW[43].

The closely relationship between obesity, T2DM and NAFLD have led into the recommendation from several experts of renaming the disease from a 'negative' term as non-alcoholic to the 'positive' term metabolic-associated fatty liver disease (MAFLD). This modification has the purpose of reflect the pathophysiology of the disease as a metabolic-driven disease, so, based on that, the presence of hepatic steatosis as well as one of the three following criteria: overweight or obesity, T2DM or metabolic dysfunction constitute the factors to diagnose MAFLD. Metabolic dysfunction is identified as the manifestation of at least two of the criteria listed in Table 2[52]. Additionally, the exclusion of other causes of liver disease such as alcohol or viral infections, which were essentially needed to NAFLD diagnosis, are not required in this new term[51]. The results provided by different authors showed that the identification of patients with high risk of disease progression is more effective and practical based on MAFLD than NAFLD criteria[53,54]. Moreover, it has been demonstrated that using the term MAFLD significantly improves the clinical utility[55]. Regarding the scientific community, the term MAFLD reflects better than NAFLD the role of metabolic dysfunction as the main driver of the disease, so it could improve the research made in the field. Contrarily, among the main proposed disadvantages about MAFLD term is highlighted the fact that this change could confuse patients and could reduce the awareness of the importance of the disease, as well as that a quick change in the terminology could impact inclusion or exclusion criteria of ongoing clinical trials with the respective consequences[51]. However, the most widespread drawback about MAFLD term is the possibility that patients with non-obese NAFLD or lean-NAFLD could be out of the focus or underestimated[51,56]. Altogether, the research points out to the fact that we are experiencing a transition time towards the implementation of a more accurate term, although more research is needed to deepen in all the possible implications and consequences of this change. In the present PhD Thesis, in order to be consistent with the

publications that endorse this work, thus avoiding possible confusion for readers, and since the proposal of changing the name to MAFLD is quite recent, the term NAFLD will be used.

Table 2. Main criteria used to define metabolic dysfunction. Adapted from [52].

Criteria defining metabolic risk factors		
Waist circumference	Caucasian popullation	≥ 102 cm ♂
		≥ 88 cm ♀
	Assian popullation	≥ 90 cm ♂
		≥ 80 cm ♀
Blood pressure	≥ 130/85 mm Hg or specific drug treatment	
Plasma triglycerides	≥ 150 mg/dl or specific drug treatment (≥ 1.70 mmol/l)	
Plasma HDL-cholesterol	< 40 mg/dl ♂ (< 1.0 mmol/l) or specific drug treatment	
	< 50 mg/dl ♀ (< 1.3 mmol/l) or specific drug treatment	
Prediabetes	i.e. fasting glucose levels 100 to 125 mg/dl, or 2-hour post-load glucosa levels 140 to 199 mg/dl or HbA1c 5.7% to 6.4%	
HOMA-IR	≥ 2.5	
Plasma high-sensitivity CRP level	> 2 mg/l	

CRP, C reactive protein; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance.

In summary, despite the significant burden of the disease in worldwide health, the global consciousness of NAFLD is scarce, with a lack of empathizing information in the health agendas of the different countries[51]. The increasing tendency of the disease, together with the absence of an effective treatment to face it, point out to the need of a multidisciplinary approach which includes health programmes, political strategies, raising social awareness and profound basic and clinical research to deal with NAFLD.

2.2. PATHOGENESIS

Non-alcoholic fatty liver disease is a multifactorial pathology with a complex and non-fully understood mechanism of development, in which genetics, epigenetics and different environmental factors lead to physiological dysfunctions that promote the origin and the progression of the disease. In 1998, James and Day proposed the 'two-hit' hypothesis to explain NAFLD pathogenesis, whereby the existence of a first hit to the liver, mainly lipid accumulation, increases the susceptibility of the organ to the damage provoked by a second hit[57]. Therefore, hepatic triglycerides accumulation due to high fat

diets, sedentarism and/or obesity, promotes a specific and prooxidant environment, in which different factors like oxidative stress, inflammatory cytokines, or mitochondrial dysfunction trigger inflammation, fibrogenesis and, consequently, NAFLD progression. However, although this hypothesis was supported by *in vivo* experiments and was the first approach that could partially explain NAFLD development, later studies demonstrated that the pathogenesis is much more complex: the parallel and synergistic action of different factors, together with genetic predisposition, are involved in the development of the disease.

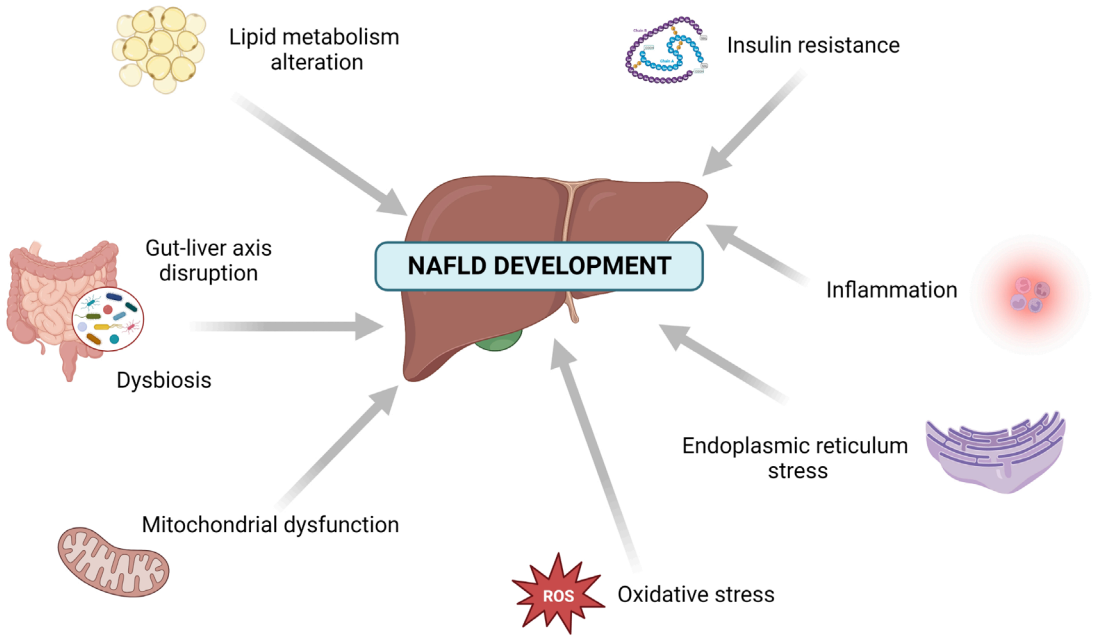


Figure 6. Pathogenesis of NAFLD: 'multiple-hit' hypothesis. Based on the 'multiple-hit' hypothesis, different factors such as lipid metabolism alteration, insulin resistance, inflammation, endoplasmic reticulum stress, oxidative stress, and reactive oxygen species (ROS) release, mitochondrial dysfunction, gut-liver axis disruption and dysbiosis could synergistically favor the development and progression of NAFLD.

For these reasons, in 2010, Tilg and Moschen suggested the 'multiple-hit' hypothesis, currently accepted, according to which, the existence of different factors can concurrently and synergistically participate in NAFLD progression. Therefore, alteration of lipid metabolism, insulin resistance, mitochondrial dysfunction and oxidative stress, endoplasmic reticulum stress and inflammation, together with dysbiosis (intestinal microbiota alteration) and gut-liver axis disruption, are the main 'hits' that could be involved in the pathogenesis of this disease (**Figure 6**)[58].

Due to the relevance of NAFLD pathogenesis for the comprehension of the present PhD Thesis, the explanation of the role of each one of the aforementioned factors is detailed below, except for the gut-liver axis disruption and the gut microbiota alteration, both factors that are described in the next chapter.

2.2.1. Alteration of lipid metabolism

NAFLD disease is characterized by the hepatic accumulation of triglycerides (TGs) derived from the esterification of free fatty acids (FFAs). This accumulation of TGs results from the imbalance between the lipid intake and the lipid consumption in the liver[44]. On one hand, there are three main sources of FFAs in the liver: diet, *de novo* lipogenesis (DNL), and lipolysis of the adipose tissue. On the other hand, the consumption of TGs in the liver is mainly made by fatty acid oxidation in the mitochondria and by the export of very-low density lipoproteins (VLDL)[59] (**Figure 7**). Therefore, this imbalance is responsible of the accumulation of TGs in the hepatocytes and, consequently, of the development of NAFLD. In fact, patients with NAFLD have high serum concentration of FFAs compared to control individuals[60]. Although the accumulation of TGs in the liver is not toxic *per se*, as it has been considered an adaptative response of the organism to an excess of energy intake, it entails a lipotoxicity status, defined as the production and accumulation of toxic lipid-derived metabolites and intermediaries, that promotes inflammation, mitochondrial dysfunction, insulin resistance and the consequent liver injury[61,62].

Firstly, regarding the lipid intake, it is estimated that 60% of the lipid accumulation in the liver comes from lipolysis, whereas 26% from DNL and 15% from the diet[44]. As was shown in previous chapters, the activation of lipolysis pathway in the adipose tissue takes place when the capacity of hyperplasia and hypertrophy of the adipocytes reaches its limit. However, it has also been demonstrated that, during NAFLD, there is a preferential accumulation of lipids in the liver rather than the adipose tissue due to the increased expression of different proteins involved in lipids uptake, such as fatty acid transporters[60]. Moreover, patients with NAFLD have an enhancement of DNL pathway, which in normal conditions is strictly regulated by different transcription factors such as sterol regulatory element-binding protein (SREBP), liver X receptor (LXR), carbohydrate response element-binding protein (ChREBP) and peroxisome proliferator-activated receptor (PPAR)-γ. SREBP has three isoforms: SREBP-1a, SREBP-1c and

SREBP-2. Hereby, while SREBP-1c regulates DNL, SREBP-2 participates in cholesterol homeostasis[61]. SREBP1-c and ChREBP promote the expression of the main enzymes involved in DNL: acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), while LXR is able to regulate not only these two enzymes but also the two transcription factors SREBP-1c and ChREBP[63]. Moreover, the role of PPAR-γ is the upregulation of different genes related to lipogenesis, triglyceride synthesis and to the formation of lipid droplets, in which *Perilipin 2* (*Plin2*) gene is involved[59].

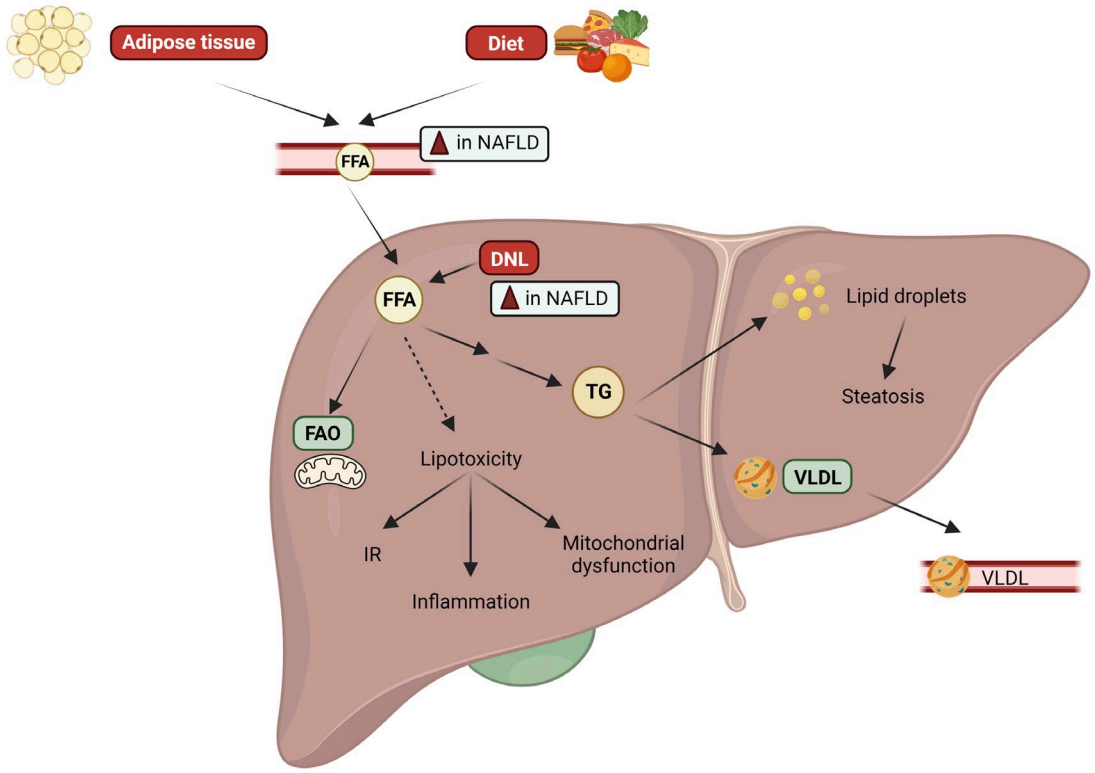


Figure 7. Hepatic lipid metabolism: role in NAFLD development. Excessive hepatic accumulation of triglycerides, known as hepatic steatosis, is one of the main characteristics of non-alcoholic fatty liver disease, and it is the result of an imbalance between the hepatic sources of lipid intake (lipolysis in the adipose tissue, diet, and *de novo* lipogenesis) and lipid consumption (fatty acid oxidation and very-low density lipoprotein export). This excessive accumulation of lipids produces lipotoxicity, leading to insulin resistance, inflammation, and mitochondrial dysfunction. DNL, *de novo* lipogenesis; FAO, fatty acid oxidation; FFA, free-fatty acids; IR, insulin resistance; NAFLD, non-alcoholic fatty liver disease; TG, triglycerides, VLDL, very low-density lipoprotein. The main sources of free-fatty acids in the liver are highlighted with a red box, whereas the main processes of lipid consumption are highlighted with a green box.

In addition to transcription factors, different enzymes also take part of lipid metabolism regulation, such as diacylglycerol O-acyltransferase 2 (DGAT2), which participates in the last step of the reaction of esterification of FFAs to TGs and whose deficiency leads to liver injury and NAFLD development[64]; or stearoyl CoA-desaturase-

1 (SCD-1), which regulates the synthesis of monounsaturated fatty acids, avoiding the accumulation of saturated fatty acids, molecules that promote endoplasmic reticulum stress and apoptosis[44].

Patients with NAFLD have an enhancement of DNL pathway, as high levels of fatty acid transporters like FATP2 and FATP5 have been reported[44]. Additionally, insulin and glucose are activators of SREBP1-c and ChREBP, respectively, so DNL is associated with an hyperglycemic and hyperinsulinemic state, both characteristics of NAFLD[44]. Altogether, during DNL, different lipid intermediates such as ceramides or diacylglycerols (DAGs) are synthetized, provoking the activation of inflammatory pathways, insulin resistance, mitochondrial dysfunction, and cell damage, worsening NAFLD development[60,62]. Lastly, the lipid intake can be enhanced by high-fat and high-carbohydrate diets, worsening NAFLD progression.

The alteration of lipid metabolism and the lipotoxicity status during NAFLD development also involve the lipid consumption processes: mitochondrial fatty acid oxidation, which is explained in next chapters, and VLDL[61]. In this sense, patients with obesity and NAFLD have shown an enhancement in the secretion of VLDL, although this mechanism is not enough to counteract the accumulation of lipids in the liver, which leads to an impairment of the process during the disease progression[59].

In brief, the accumulation of lipids in the liver due to different reasons such as sedentarism, high-fat or high-carbohydrate diets leads to an alteration of the lipid metabolism and to a state of lipotoxicity that not only aggravates NAFLD development but also induces mitochondrial dysfunction, oxidative stress, insulin resistance, endoplasmic reticulum stress and inflammation, factors that, as it is explained below, also participate in the worsen of the disease' pathogenesis.

2.2.2. Insulin resistance

Insulin resistance (IR), defined as a reduction in the systemic physiological response to insulin signaling, is a key contributor in NAFLD development. Despite decreased insulin sensitivity affects the whole body, adipose tissue, skeletal muscle, and liver are the main tissues involved in this process[65].

Firstly, in adipose tissue, the main role of insulin in normal conditions is promoting lipogenesis and inhibiting lipolysis. However, an insulin-resistant adipose

tissue is characterized by the activation of lipolysis, with the associated release of FFAs to the systemic circulation and the consequent ectopic fat accumulation, mainly in the liver, visceral adipose tissue, and skeletal muscle[66]. In this sense, patients with NAFLD have higher blood FFAs levels, variable that has been positively correlated with NAFLD development[59].

Secondly, to comprehend the role of insulin in the liver, is needed to know that the main proteins through the insulin signaling takes place are the insulin receptors substrate 1 and 2 (IRS-1, IRS-2). The binding of insulin to its receptor activates IRS signaling, which consequently activates glucose intake, glycogen synthesis and lipogenesis, whereas inhibits gluconeogenesis and lipolysis[67]. Therefore, in the liver insulin stimulates DNL through the activation of SREBP-1c by IRS-2 signaling. In a condition of insulin resistance, the expression of IRS-2 is downregulated, fact that leads to an overexpression of SREBP-1c and the subsequent increase in DNL, contributing to NAFLD development[65]. Moreover, the FFA infusion from lipolysis process in adipose tissue and the following accumulation of lipids such as diacylglycerols (DAGs) or ceramides in the liver also contribute to the impairment of the insulin signaling and glucose uptake, favoring hepatic gluconeogenesis, insulin resistance and worsening NAFLD[61,66]. In summary, there is a lack of inhibition of the glucose production whereas there is an induction of the DNL in the liver, both processes in which insulin resistance is involved[66].

Furthermore, in physiological conditions, insulin promotes the activation of the phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway in the skeletal muscle, which lets the glucose to be absorbed by the cells. Depending on the energetic status, the glucose can be stored as glycogen or used to produce energy through adenosine triphosphate (ATP) synthesis. Hereby, this process is significantly impaired in an insulin-resistant condition, since there is not only an impaired IRS-1 pathway that reduces the glucose uptake, but also a defective glycogen synthesis[65].

Finally, it is worth mentioning that, on one hand, different genetic and environmental factors could interfere and favor insulin resistance[61], and, on the other hand, insulin resistance could be also promoted by other factors that are involved in NAFLD development, as it is explained below in each section.

2.2.3. Mitochondrial dysfunction and oxidative stress

The alteration of the function and the structure of the mitochondria, the main organelle in which the oxidation of the FFAs takes place, is not only a contributor but also a consequence of NAFLD development[60,68]. Mitochondria is an organelle characterized by its ability to catabolize lipids and carbohydrates through β-oxidation, tricarboxylic acid cycle (TCA), oxidative phosphorylation (OXPHOS) and ketogenesis[37]. To begin with, the FFAs influx that comes from lipolysis, diet or even *de novo* lipogenesis induces an increase of the fatty acid oxidation (FAO), TCA cycle and OXPHOS processes in the organelle in order to maintain the lipid turnover and to avoid lipotoxicity[37,44] (**Figure 8**). In this sense, patients with NAFLD have shown a higher activity of FAO and of the TCA cycle[59,60,69,70]. However, the excessive hepatic accumulation of these FFAs overwhelms the mitochondrial capacity to process them, impairing the beta-oxidation and leading to different consequences that altogether promote NAFLD development, as it is summarized in **Figure 8**. Firstly, the alteration of FAO process is associated with an imbalance in the lipid homeostasis and the resulting synthesis of lipid-derived toxic metabolites, mainly ceramides and DAGs that, as it was previously mentioned, not only contribute to insulin resistance, inflammation, and fibrosis, but also favor mitochondrial dysfunction through the inhibition of the electron transport chain[37,44]. In this sense, mitochondria have different functions more than energy production and lipid metabolism, since they participate in urea cycle, iron metabolism and regulation of inflammation and immune system[68], so its dysfunction significantly alters physiological homeostasis and contributes to disease progression. Moreover, the mitochondrial DNA that is released as consequence of the damage induced by the lipid intermediaries in the hepatocytes constitutes a proinflammatory molecule that promotes inflammation, and consequently the progress from NAFLD to NASH[44]. Additionally, due to mitochondrial dysfunction, there is a decrease in the mitophagy process, promoting the accumulation of altered mitochondria in the cell that leads to cell necrosis, inflammation and NAFLD development[68].

Secondly, this alteration is also related to an overproduction of reactive oxygen species (ROS), contributing to oxidative stress, one of the main causes of hepatocytes death[71]. The oxidative stress takes place when an imbalance between the production of ROS and the antioxidant capacity of the organism is produced in favor of the first[72]. In the mitochondria, the continuous supply of FFAs to the electron transport chain promotes

an increase in the ROS production. However, other different sources of ROS have also been identified apart from the mitochondria, such as microsomes, peroxisomes and enzymes in the plasmatic membrane. Regarding peroxisomes, organelles responsible of the oxidation of long-chain FFAs, an impaired peroxisomal β-oxidation and an overexpression of the peroxisome proliferator-activated receptor-α (PPAR-α), a transcriptional factor involved in the lipid metabolism control, have been related to hepatic injury[44]. Additionally, the detoxification mechanism in the liver also produces ROS, so besides the excessive FFAs influx, the presence of toxins could lead to oxidative stress, promoting tissue damage trough enhancement of inflammatory status pathways[72]. In health-related conditions, the antioxidant machinery of the mitochondria and, in general, of the whole cell is able to counteract ROS. However, an overproduction of ROS has been demonstrated to overwhelm and inhibit some of the antioxidant mechanisms of the organism, such as the enzymes superoxide dismutase (SOD) or the molecule glutathione[64]. In fact, patients with NAFLD have shown an enhanced oxidative stress and decreased levels of vitamin E, an antioxidant molecule, showing the relationship between the disease and the presence of oxidative stress[72]. ROS and the consequent oxidative stress, apart from the mentioned damaged effects, also aggravate mitochondrial dysfunction and promote the lipid peroxidation of polyunsaturated FFAs, producing high-reactive and toxic metabolites such as malondialdehyde[64].

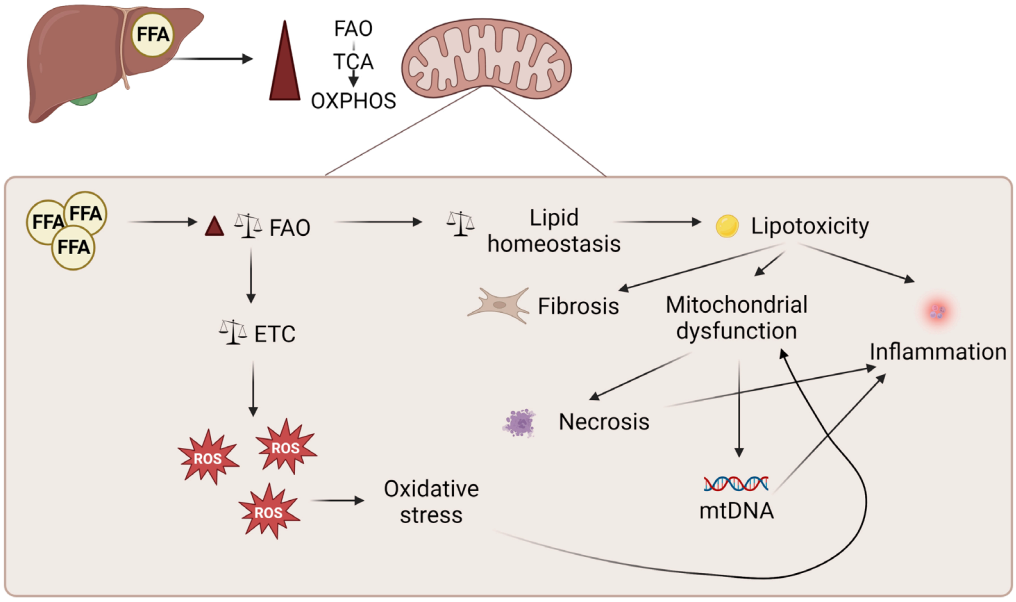


Figure 8. Mitochondrial dysfunction in NAFLD pathogenesis. The excessive accumulation of free-fatty acids in the liver induces an increase in the mitochondrial activity, mainly in fatty acid oxidation, tricarboxylic acid cycle and oxidative phosphorylation, which overwhelms the mitochondrial capacity to process these molecules, leading to an imbalance of the mitochondrial electron transport chain with the

consequent release of reactive oxygen species and oxidative stress, and an imbalance of lipid homeostasis, which induces lipotoxicity and, consequently, fibrosis development, inflammation and mitochondrial dysfunction. ETC, electron transport chain, FAO, fatty acid oxidation; FFA, free-fatty acids; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle.

It is also worth it to mention the role of nicotinamide adenine dinucleotide (NAD) metabolism in NAFLD development. NAD is a hydride acceptor that allows the hydrogen transfer during metabolic reactions, so is an essential molecule on FAO, TCA or OXPHOS in the mitochondria. Since the concentration of NAD is regulated by the energetic status, an excess of caloric intake reduces NAD levels[73]. In this sense, the nicotinamide phosphoribosyltransferase (NAMPT) is a protein regulator of NAD levels, as catalyzes the limit step of the reaction, and also controls the activity of sirtuins, NAD-dependent proteins involved in mitochondrial biogenesis and in the response to different stresses like oxidative stress or inflammatory stress[73,74]. NAD metabolism has been identified to protect from NAFLD development, as NAD deficiency, which has been related to a downregulation of NAMPT expression, promotes a decrease in FAO which consequently leads to the hepatic FFA accumulation. Moreover, NAMPT activates NAD synthesis, fact that activates SIRT1 and SIRT3, both molecules able to alleviate hepatic steatosis[73]. In fact, SIRT1 inhibits SREBP1, a transcriptional factor involved in lipogenesis induction and low levels of SIRT1 have been related to NAFLD pathogenesis[74]. Altogether, NAD metabolism, as well as NAMPT and sirtuins seem to be also involved in NAFLD pathogenesis.

In summary, the mitochondrial dysfunction caused by the excessive influx of FFAs together with an alteration of NAD metabolism in the liver lead to an incomplete process of fatty acid oxidation, producing not only toxic metabolites but also enhancing insulin resistance, ROS synthesis, oxidative stress and inflammation[60], all processes that promote liver damage and NAFLD development[59,66].

2.2.4. Endoplasmic reticulum stress

The endoplasmic reticulum (ER), widely present in hepatocytes, is the responsible organelle of different physiological functions such as protein folding, calcium homeostasis, vesicular transport, and lipid biosynthesis[75]. In order to solve the ER stress produced by different factors, this organelle counts with the 'unfolded protein response' (UPR), through which the reduction of protein synthesis, the improvement of protein

transport, the removal of unfolded proteins that have been accumulated or even the apoptosis of the cell itself are performed based on the grade of stress[61,75]. In physiological conditions, the UPR response relies on three different pathways, which are induced by the activation of three different transmembrane proteins: the Activating Transcription Factor 6 (ATF6), the Inositol-Requiring Enzyme 1 (IRE1) and the protein kinase RNA-Like ER kinase (PERK). These three proteins are sensors of the ER stress and are inactivated due to their binding to the glucose regulated protein (GRP) 78/Binding immunoglobulin protein (BiP), until the stress threshold is reached[38]. In NAFLD, different factors such as oxidative stress, mitochondrial dysfunction, hepatic lipid accumulation or hyperglycemia are able to activate the UPR response, contributing to overwhelm ER stress signaling in the three of the different pathways[75]. However, this process is like a treadmill, due to ER stress also leads to insulin resistance, apoptosis, hepatic *de novo* lipogenesis and ROS release, worsening NAFLD development[59]. Additionally, the role of ER stress in lipid metabolism also contributes to NAFLD disease. In fact, Kim *et al.* showed that ER stress causes the accumulation of TGs and cholesterol in the hepatocytes through the regulation of SREBP1 and SREBP2, respectively, favoring NAFLD to NASH progression[76].

Altogether, the role of ER stress in the development of NAFLD has been briefly summarized, highlighting the bidirectional link between other factors involved in the pathogenesis of the disease, such as oxidative stress or lipotoxicity, that promote ER stress and the ER stress itself, which leads to the appearance of these and other NAFLD-related factors. Therefore, this vicious circle contributes together to the progression and development of NAFLD.

2.2.5. Inflammation

During NAFLD development, hepatic steatosis could progress to steatohepatitis (NASH), characterized by inflammation and hepatocytes damage. Inflammation, as it was previously explained, is promoted by different and diverse factors such as lipid-derived metabolites, mitochondrial dysfunction, or ROS production, among others. However, proinflammatory factors related to NAFLD development have been traditionally classified into hepatic and extrahepatic factors[77].

Among the first ones, summarized in **Figure 9**, the lipotoxicity induced by the hepatic accumulation of FAs, together with the presence of lipid-derived intermediates

such as ceramides or DAGs, induce the expression of the transcription factor NF-κB, which promotes the synthesis of different proinflammatory molecules such as tumor necrosis factor (TNF), or interleukins (IL) like IL-1β or IL-6. TNF not only enhances the expression of the NF-κB, but also activates the c-Jun N terminal kinase (JNK) pathway, both proinflammatory pathways[25]. Additionally, the released interleukins contribute to the recruitment of hepatic macrophages, called Kupffer cells, which mediate the inflammatory response by stimulating the secretion of more proinflammatory cytokines, the infiltration of other immune cells like monocytes and the activation of hepatic stellate cells (HSC)[37,50]. In this sense, macrophages and HSC produce chemokines as C-C motif chemokine ligand 2 (CCL2) or C-X-C motif chemokine ligand 10 (CXCL10), which are recognized by C-C motif chemokine receptors (CCRs) like CCR-5 in order to recruit more immune cells to the liver[78]. Furthermore, Kupffer cells are also able to inhibit IRS-1 and IRS-2, promoting insulin resistance[66], and to induce the secretion of profibrogenic molecules, such as transforming growth factor-β (TGF-β), that is responsible of the fibrogenic process that leads the progression from NAFLD to NASH and to advanced fibrosis[66,79]. In that regard, fibrosis is defined as the accumulation of extracellular matrix proteins that leads to the deposition and the scarring of the tissue[77], so the activation of HSC and their ability to secrete extracellular matrix components are the main factors involved in the profibrogenic process[37,77]. Related to that, HSC activation increases the expression of α-smooth muscle actin (α-SMA), metallopeptidases (MMP) like MMP-3 and MMP-9, collagen type I by the activation of the collagenase enzymes such as Col1A1, and TGF-β, favoring fibrosis development[80].

Together with the NF-κB and JNK proinflammatory pathways, the inflammasomes also play an essential role in NAFLD-related inflammation. Inflammasomes are multi-protein and cytoplasmic complexes that act as sensors of damage-associated molecular patterns (DAMPs) or pathogen-associated molecular pattern molecules (PAMPs)[61]. Therefore, the molecules that are released by the damaged hepatocytes, such as DNA, RNA or proteins, as well as the presence of FFAs or lipid intermediaries such as ceramides or DAGs are recognized as DAMPs by pattern recognition receptors (PRRs) presented in Kupffer cells, hepatocytes, HSC, and dendritic cells[37,79]. The main PRRs are the Toll-like receptors (TLR), which are able to activate different inflammasomes and potentiate the inflammatory signaling in NAFLD development[79]. Inflammasomes also activate HSC, favoring fibrosis progression[61]. The

NLR family pyrin domain containing 3 (NLRP3) inflammasome is the most related to NAFLD development inflammasome and its activation leads to the secretion of the proinflammatory cytokines IL-1β and IL-18, that further promote inflammation and fibrosis[77,79,81]. The immune cells recruited in the liver not only synthetize proinflammatory cytokines and chemokines, but also eicosanoids and ROS, aggravating the inflammatory status[79]. Moreover, inflammasomes also involved in the regulation of pyroptosis too, a cell death process whose release of intracellular material aggravates inflammatory status[77].

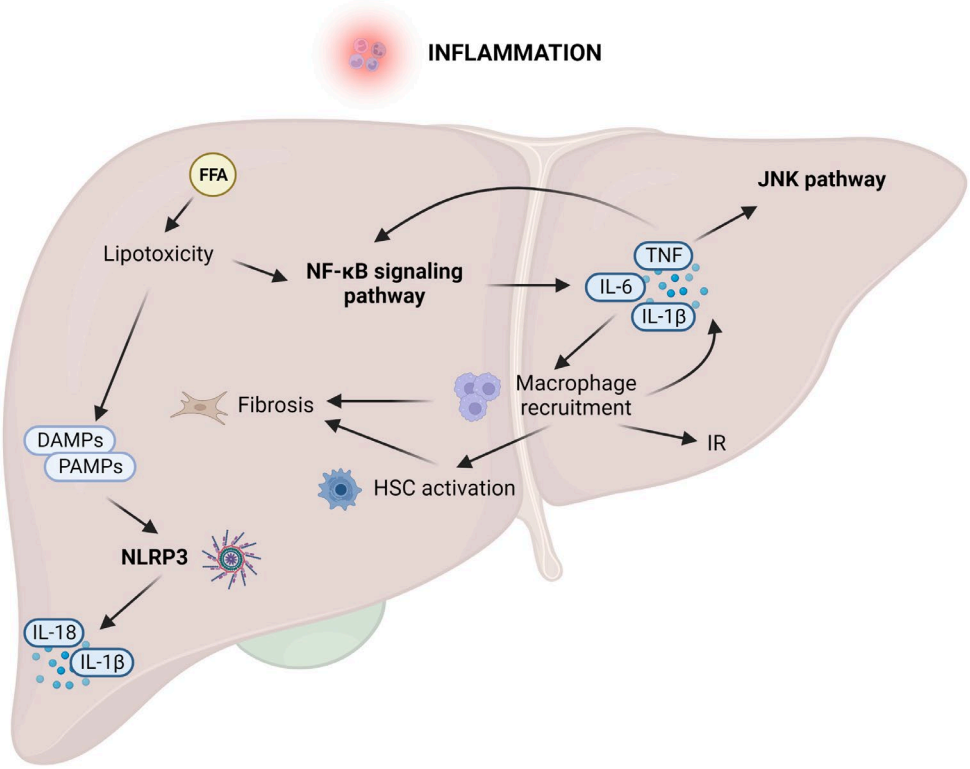


Figure 9. Inflammation in NAFLD pathogenesis. Excessive hepatic accumulation of free-fatty acids leads to a status of lipotoxicity, which promotes the activation of the main proinflammatory pathways: NF-κB pathway, JNK pathway and NLRP3 inflammasome pathway, with the consequent release of proinflammatory cytokines and the activation and recruitment of immune cells, aggravating the inflammatory status and favoring NAFLD development. DAMPs, damage-associated molecular patterns; FFA, free-fatty acids; HSC, hepatic stellate cell; IL, interleukin; IR, insulin resistance; JNK, c-Jun N terminal kinase; NF-κB, Nuclear factor kappa B; NLRP3, NOD-like receptor family pyrin domain containing 3; PAMPs, pathogen-associated molecular patterns; TNF, tumor necrosis factor.

Regarding the main extrahepatic factors that promote inflammation, diet could constitute a proinflammatory stimulus, as Western diets have been related not only to inflammation *per se* but also to the alteration of the gut microbiota that, as it is described below, is also associated with inflammation[77]. Moreover, adipose tissue, as formerly

explained, is able to synthetize different proinflammatory adipokines such as TNF or interleukin-6 (IL-6) that not only favor lipolysis but also promote insulin resistance and systemic inflammation, with the consequent NAFLD progression[59,81]. Leptin and adiponectin, the main hormones secreted by the adipose tissue, which have been detected in high and low levels, respectively, in patients with NAFLD, also play a role in the disease[82,83]. Leptin is an anorexigenic hormone that has a profibrogenic role due to its ability to activate hepatic stellate cells (HSC), whereas adiponectin has shown anti-inflammatory, antifibrotic and hepatoprotective effects[61]. Therefore, the proinflammatory mediators and hormones secreted by the adipose tissue amplify the inflammation and aggravate NAFLD[78].

In summary, different hepatic and extrahepatic factors such as Western diet, alteration of the gut microbiota, adipose tissue dysfunction, hepatic lipotoxicity, mitochondrial damage, ER stress, or ROS production contribute to the induction and progression of the inflammatory and fibrotic status that aggravate NAFLD development.

2.3. Diagnosis of NAFLD

Currently, there are different diagnostic tools for NAFLD, including invasive and non-invasive methods. Liver biopsy is nowadays the gold-standard technique for NAFLD diagnosis, constituting the most precise available strategy[39,49]. Liver biopsy allows to perform a histological evaluation of liver status, commonly based on the NAFLD Activity Score (NAS), a standardized method that consists in assigning a score based on the total sum of the evaluation of three parameters: steatosis degree (graded from 0 to 3), inflammation (graded from 0 to 3) and ballooning (graded from 0 to 2). Therefore, a total score equal or greater than 5 correspond to NASH diagnosis[84,85]. However, liver biopsy is expensive, significantly invasive, and has related risks and complications[40,49]. Therefore, even though biopsy remains the conclusive tool for NAFLD diagnosis, it cannot be considered as a screening method[49].

For those reasons, other strategies have been developed and widely implemented in the last years. In this sense, imaging techniques are one of the most important non-invasive current approaches. Herein, ultrasonography is the most used tool due to its low cost, easily performing and its sensitivity for detection of moderate and severe steatosis[86,87]. However, this strategy has different limitations, such as low sensitivity for minor steatosis and for patients with obesity, as well as the subjectivity[86].

Moreover, it cannot be used to diagnose fibrosis and it is not a quantitative test[87]. Consequently, improved technologies based on ultrasonography, but including quantitative approaches were developed. Controlled attenuation parameter (CAP) is a validated tool to diagnose hepatic steatosis, including mild steatosis, with a high interobserver reproducibility, but with the main limitation of measurement failure. The widely known Fibroscan® is a commercial equipment based on this methodology combined with elastography[40,86]. Additionally, magnetic resonance-based techniques are also used in NAFLD diagnosis, being one of the most sensitive methods[87]. Among them, magnetic resonance imaging (MRI)-derived proton density fat fraction (MRI-PDFF) is considered as the most accurate imaging tool for the quantification of hepatic steatosis, with the advantage that has no problems with NAFLD diagnosis in patients with obesity. However, the main limitations of MRI tools are the cost, the complexity, and the limited availability, needing specific equipment and qualifying operators[86,88].

Recently, different serum biomarkers have been proposed for NAFLD and NASH diagnosis. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) serum levels are commonly increased in patients with NAFLD but are not indicators of the stage of the disease and a high percentage of NAFLD patients have normal levels of these enzymes, reasons why they are not considered good biomarkers[40]. Serum concentration of cytokeratin-18, a protein released during apoptosis of hepatocytes, is correlated with the severity of NASH. Additionally, inflammatory markers such as the cytokine C-C-C motif chemokine ligand 10 (CXCL10), tumor necrosis factor (TNF) or interleukin 8 (IL-8), as well as adipocytokines and hormones like fibroblast growth factor 21 (FGF21) have been also proposed for NASH diagnosis[88]. Although biochemical markers are cheap and simple, they are not as reliable as imaging techniques or liver biopsy, so biomarker panels that combine biochemical parameters with other measurements like body mass index have been also developed in order to improve the accuracy and specificity of the diagnosis. Fatty liver index (FLI), hepatic steatosis index (HSI), or SteatoTest are the panels focused on NAFLD diagnosis, whereas NASHTest or NASH ClinLipMet score are the ones oriented to NASH identification[40,88]. However, each one has its own disadvantages and most of them are not fully validated in all NAFLD cohorts.

Overall, the diagnosis of NAFLD is under discussion because of the high probabilities of its underestimation based on the diagnostic tool used. For example, the

prevalence of NAFLD in US was estimated in 24.13% by ultrasonography, but this data downed to 21.09% using the blood testing[43]. Moreover, despite the available techniques, NAFLD is widely underdiagnosed, also because of the absence of symptoms until advanced stages[49,89]. Due to the high prevalence of NAFLD and the consequent rates of morbidity and mortality, an accurate diagnosis of the disease in the early stages is needed to avoid the progression and resulting economic and social burden. In this sense, it has been reported the importance of establishing a screening program to identify patients with higher risk of NAFLD progression, such as patients with obesity or patients with T2DM[89]. Currently, more efforts are involved in developing new strategies, as the ones focused on lipidomics and metabolomics as emerging omics with promising, but preliminary and not yet validated results[90].

2.4. TREATMENTS

Nowadays, there is no available treatment for NAFLD. The medical primary recommendations for facing the disease are changing the lifestyle towards a healthy pattern, including specific diets and practice exercise[91]. Firstly, what is certain is that NAFLD patients have a common dietary pattern, characterized by the intake of high amounts of sugars and saturated fats, refined grains, as well as low intake of fruits, vegetables, cereals, and whole grains. Therefore, as nutrition is an important factor that influences NAFLD development, dietary intervention is the first step to try to manage the disease[92]. Herein, the main recommendation is the gradual weight loss through energy restriction: while the American Association for the Study of Liver Diseases (AASLD) suggests a reduction of 3-5% of total body weight, the European Association for the Study of the Liver (EASL) recommends a reduction of 7-10%. In fact, a reduction of 3-5% is able to improve hepatic steatosis, while a loss of 10% of body weight has been reported to significantly improve steatosis, inflammation, and fibrosis[48,88]. In order to achieve this goal, different nutritional strategies have been postulated due to its benefits in NAFLD patients, such as low-carbohydrate diet, low-fat diet, ketogenic diet, or Mediterranean dietary pattern[92]. Independently of the nutritional plan chosen, the main challenge is to maintain the adherence at long term, as well as to avoid body weight regain[88]. In this sense, a meta-analysis of 29 studies determined that approximately 80% of the lost weight was regained within the following five years[93]. Recently, intermittent fasting, defined as a nutritional intervention in which there are intervallic periods of abstinence from food

and energy-containing fluids, could be a feasible alternative. Preclinical studies have demonstrated benefits such as weight loss, improvement in plasma lipid levels, as well as in insulin resistance, and decrease of inflammatory status. However, clinical trials are needed to elucidate the pros and drawbacks of the strategy[42].

Nevertheless, an approach uniquely based on the diet is not able to modify liver enzymes, the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), or the lipid profile and consequently to completely reverse the disease[94]. Therefore, physical activity is not only essential to lose body weight, but also needed to improve hepatic steatosis, endothelial function, cardiorespiratory fitness, insulin sensitivity and dyslipidaemia. Moreover, it reduces systemic inflammation, arterial blood pressure and the risk of sarcopenia[95]. Although there is no consensus about the best option for NAFLD patients in terms of type, duration and intensity of exercise, general recommendations suggest 150 minutes of moderate aerobic exercise together with strength and endurance training between two and three times per week[94]. A recent meta-analysis carried out by Zou *et al*. clarified the importance of combine practicing exercise with diet interventions, being this combinatory strategy more effective than each one alone[96]. Altogether, the combination of diet and physical activity is the main approach to face NAFLD. However, the specific dietary regimens or the exercise plans are not clear[39,92,94], being necessary to carefully individualize the treatment based on the patient characteristics, trying to avoid the loss of adherence and, consequently, the failure of the procedure.

Regarding the pharmacological approach, as it was aforementioned, there is no current medical treatment approved for facing NAFLD, excluding those aimed to treat its risk factors[97]. However, due to its high relationship with T2DM and obesity, some anti-obesity and anti-diabetic drugs, always in combination with lifestyle interventions, have been proposed as an alternative[41,48]. In this sense, there are a lot of group of drugs that have been considered for NAFLD treatment that could be scheduled based on their target in the organism: carbohydrate and lipid-metabolism targets, such as farnesoid X receptor (FXR) agonists, peroxisome proliferator-activated receptor (PPAR) agonists, glucagon-like peptide (GLP) receptor agonists or thyroid hormone receptor β (THR-β) agonists; lipotoxicity and cell death-based targets, such as vitamin E or silymarin; inflammation-based targets and extracellular matrix deposition anti-fibrosis-based targets[87].

Among the most promising strategies, it is worth mentioning that glucagon-like peptide (GLP)-1 receptor agonists. GLP-1, as it was previously mentioned, is an intestinal

hormone responsible of the stimulation of insulin production and release, so it is also responsible of the consequent inhibition of glucagon production and the reduction in food intake[98]. Based on that, GLP-1R agonists are anti-diabetic drugs that have demonstrated to reduce cardiovascular risk, diminish hepatic steatosis and liver damage[41]. The main drugs of this group are liraglutide and semaglutide, being the latter an up-and-coming alternative since its ability to reduce NAFLD with safety and efficacy, although larger trials are still needed[87]. Additionally, it cannot be forgotten to mention pioglitazone, an antidiabetic drug and a PPAR agonist that has anti-inflammatory and anti-fibrotic effects and, according to the guidelines, it can be used in patients with NASH independently of the presence of T2DM. However, one of the common side-effects of pioglitazone is body weight gain, so its use should be carefully considered in patients with NAFLD and obesity[48,87]. Additionally, the combination therapy with two or more strategies is also under the focus due to its ability to improve rates of NAFLD and NASH resolution[87,99]. Altogether, these are only a few examples of the number of drugs that are now being under research, with many of them in phase II and III of clinical trials[97], but with an undefined and unestablished approved guidelines for NAFLD.

For the aforementioned reasons, considering the challenge of the low adherence on diet regimens and physical exercise of patients and the lack of an efficient drug, and taking into account the complex pathophysiology of the disease and the multiple factors that contribute to its development, more research as well as economical and personal efforts are needed in order to achieve a safe and effective treatment.

In the last years, gut microbiota has emerged as one of the main contributor factors to the development of different metabolic diseases, such as obesity, NAFLD or even T2DM. Its importance and relevance have led to the search of new therapeutic alternatives based on gut microbiota modulation. In the next chapter, an extensive review of the gut microbiota, its functions, its composition, and modulatory factors, as well as its relationship with obesity and NAFLD is specified.

3. GUT MICROBIOTA

3.1. Gut Microbiota: Definition and Basic Concepts

Microorganisms are present in every single human niche that has been analyzed: the skin, the oral cavity, the gastrointestinal tract and even the lungs, among others[100].

The most studied human microbiome, defining microbiome as the community of microorganisms that habit in a specific niche[101], is that of the gut and constitutes the well-known called 'gut microbiota'. Based on that, it is easy to guess that the gut microbiota is the ecosystem constituted by the group of microorganisms that habit along the gastrointestinal tract. Although the bacteria are the main microorganism present in the gut microbiota, archaea, virus, and some fungi are also part of it, establishing altogether a symbiotic relationship with the host[102]. Therefore, these microorganisms use food-derived compounds that the human enzymes cannot digest, as well as dead cells and mucus from the gut to promote their proliferation, and, at the same time, produce a huge number of beneficial substances for the human organism and participate in the modulation of the immune system[103]. Although bacteria are the major microorganisms in the gut microbiota, likewise the most studied and the protagonists of the present PhD Thesis, the virome, the phageome and the mycobiome could offer another field to investigate the relationship and interactions between the host and the microbiota[104].

Such is the importance of the gut microbiota that is considered an active organ, with one hundred times larger of microbial genes compared to the human genome[105], and a ratio of microbial to human cells of approximately 1:1[106]. Additionally, and in contrast with the myth of 1.5 kg, it is estimated that 200 g of our total body weight belong to the gut microbiota[107].

In the last years, with the scientific progress and the development of new analytical techniques, the knowledge about gut microbiota has dramatically increased, delving into its functions, composition and role in health and disease. In fact, the multiple functions of the gut microbiota help to maintain the physiological homeostasis[108], so the alteration in its composition and/or functionality, called dysbiosis, are related with many diseases[104,108,109]. Obesity, metabolic syndrome, diabetes mellitus, inflammatory bowel disease, cancer, autoimmune diseases or neurodegenerative diseases are only a few examples of the number of pathologies that have been associated with gut microbiota alteration via gut-liver axis, gut-brain axis, gut-vascular axis or gut-bone axis, among others[110–112]. For those reasons, understanding the insights of this 'alive organ' could help not only to better comprehend the pathophysiology of the diseases, but also to find therapeutic alternatives.

In this chapter, it is explained and summarized the main characteristics of human gut microbiota, including its functions, its composition along life, its contribution to the

'gut-liver axis', and its role in the development of metabolic diseases, specially obesity NAFLD.

3.2. FUNCTIONS

The gut microbiota, as it was aforementioned, carries out a variety of functions in order to maintain the physiological homeostasis (**Figure 10**). Besides the role that the microbiota plays preserving the structure of the gut by the improvement of the surface of absorption, the promotion of the villi cells renewal or the increase of the intraluminal content[105], its functions could be divided into three different groups: metabolic, immunological, and neurological functions.

- **Metabolic functions.** The gut microbiota is able to process different compounds that are indigestible by the human enzymes, such as polysaccharides or polyphenols, and also plays a role in the metabolism of carbohydrates, proteins, and bile acids (BAs)[111]. In fact, gut microbiota is able to extract approximately 10-30% of the energy from the nutritional compounds[113]. Firstly, the fermentation of carbohydrates, mainly dietary fibers and complex polysaccharides, by the gut microbiota produces gases (H_2, CO_2, CH_4), methanol, ethanol, and short-chain fatty acids (SCFAs), important molecules that regulate lipogenesis and glucose metabolism, as well as constitute a source of energy production, participate in immune regulation and work as signaling molecules[114,115]. Furthermore, the non-digested proteins are metabolized by the proteases and peptidases of the gut microbiota producing peptides, branched SCFAs such as isobutyrate or isovalerate, amino acids, amines, phenol-derived compounds like indole, and ammonia[113,116]. Among the amino acids that are synthetized by the gut microbiota, it is important to mention leucine, isoleucine, valine, or glycine, being the latter essential to obtain glutathione, one of the most important antioxidant molecules in our organism[109]. Furthermore, carnitine and choline are transformed into trimethylamine (TMA) by the gut microbiota, regulating the hepatic triglyceride accumulation[116]. Regarding the lipids, gut bacteria regulate not only the lipid metabolism, but also the lipid composition as some of the bacterial species are involved in the biosynthesis of sphingolipids[114]. The gut microbiota plays a key role in the production of different vitamins too, such as vitamin C, vitamin K, thiamine, riboflavin, and folate, among others[114,117]. Additionally, intestinal microbiota has a vital function in BAs metabolism since it transforms primary BA into

secondary BA through three different types of biochemical reactions: deconjugation, dihydroxylation, epimerization and conjugation[115,118].

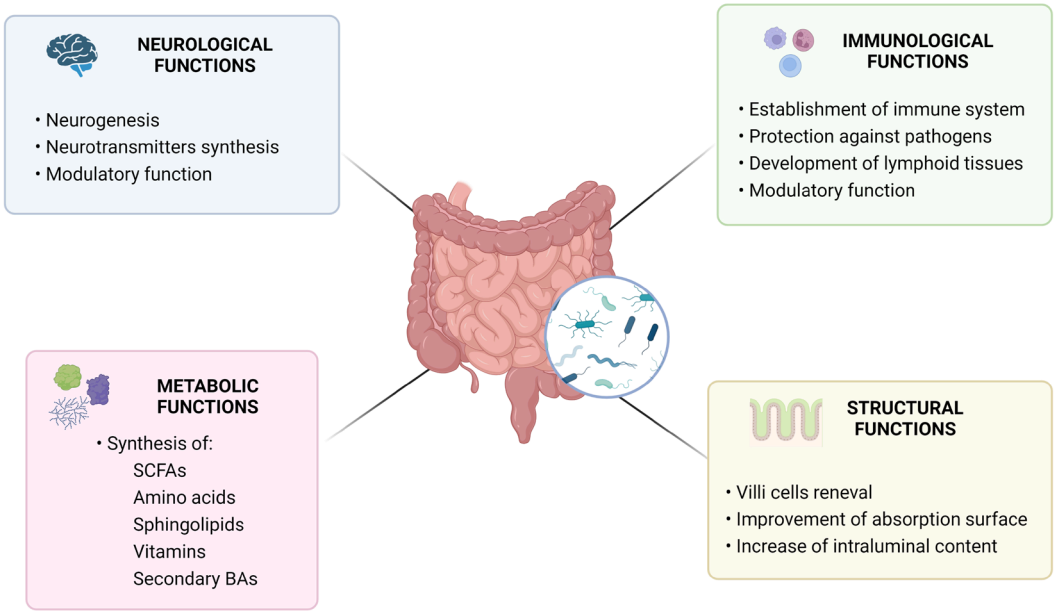


Figure 10. Schematic representation of the main functions of the gut microbiota. The main functions of the human gut microbiota in the organism are summarized in neurological, immunological, metabolic, and structural functions. SCFAs, short-chain fatty acids; BAs, bile acids.

- **Immunological functions.** The gut microbiota significantly participates in the modulation of immune system and confers protection against pathogens to the organism[119]. In fact, germ-free mice have an impaired immune system, characterized by a decrease in the production of immunoglobulin A, high levels of systemic immunoglobulin E and decreased production of antimicrobial peptides, effects that, all of them, can be restored by faecal microbiota transplantation from mice or human donors[120]. Regarding the immunological functions of the gut microbiota, on one hand, during the early stages of life, the recognition of the microbes of the gut as well as of their derived compounds let the immune system to distinguish between self and non-self-microorganisms, an essential fact in the establishment of the immune system[109]. Moreover, the development of lymphoid tissues are partially controlled by the gut microbiota, which is also able to regulate the levels of circulating innate cells[121]. Concerning microbial-derived metabolites, they have the ability to stimulate different immune cells in order to synthetize interleukins and cytokines and contribute to the hematopoiesis process[109,121]. On the other hand, gut microbiota is able to protect our organism against pathogens by different mechanisms such as the synthesis of

antimicrobial molecules, bacteriophages release, and the competition for nutrients in the niche[122,123].

- **Neurological functions.** Although the discovery of the role of gut microbiota in the nervous system is quite recent, it has helped to increase the knowledge of the physiology of our body. The gut microbiota is involved in a bidirectional relationship with the enteric nervous system (ENS), the central nervous system (CNS) and the endocrine system, called 'gut-brain axis', and participates in the brain development and neurogenesis. In fact, germ-free animals have different neurological-related alterations such as visceral pain, neurodegenerative diseases, or depression[124]. Gut microbiota is able to produce different types of neurotransmitters, such as serotonin, catecholamines, tryptophan and gamma-aminobutyric acid (GABA)[123], as well as to modulate the enteric sensory afferents or produce different metabolites with a modulatory role at neural level. It is worth mentioning that approximately a half of the dopamine and the 90% of the serotonin of our body, both neurotransmitters involved in the control of mood, happiness, or pleasure feelings, are produced in the gut[113]. Additionally, gut microbiota, as it was aforementioned, stimulates the production of cytokines, some of which are able to regulate the neurological function and consequently produce changes in the behavior or in the mood[117].

Finally, gut microbiota also promotes the intestinal absorption of Ca, Mg, P and Fe minerals, regulates bone density through the osteoclast-osteoblast mediated processes, and helps to detoxify different xenobiotics[105,117,125].

3.3. Composition and Key Modulatory Factors

Human gut microbiota is made up of permanent and transitory bacteria that mostly belong to five different phyla: Firmicutes (64%), Bacteroidetes (23%), Actinobacteria (3%), Proteobacteria (8%) and Verrucomicrobia (<2%)[109,122]. Based on the recent announcement made by the International Committee on Systematics of Prokaryotes (ICSP)[126], the nomenclature of forty-two phyla of prokaryotes has been changed. Although these changes are included in the present section (**Table 3**), this PhD Thesis keeps the previous nomenclature in order to be consistent with the published articles that endorse and constitute it.

Table 3. Relation between the former and the new nomenclature proposed by the ICSP of the main phyla of gut human microbiota.

Former nomenclature	New nomenclature
Firmicutes	Bacillota
Bacteroidetes	Bacteroidota
Actinobacteria	Actinomycetota
Proteobacteria	Pseudomonadota
Verrucomicrobia	Verrucomicrobiota

ICSP, International Committee on Systematics of Prokaryotes.

The composition of gut microbiota varies not only throughout the whole life (**Figure 11**), but also along the gastrointestinal tract of an individual (**Figure 12**).

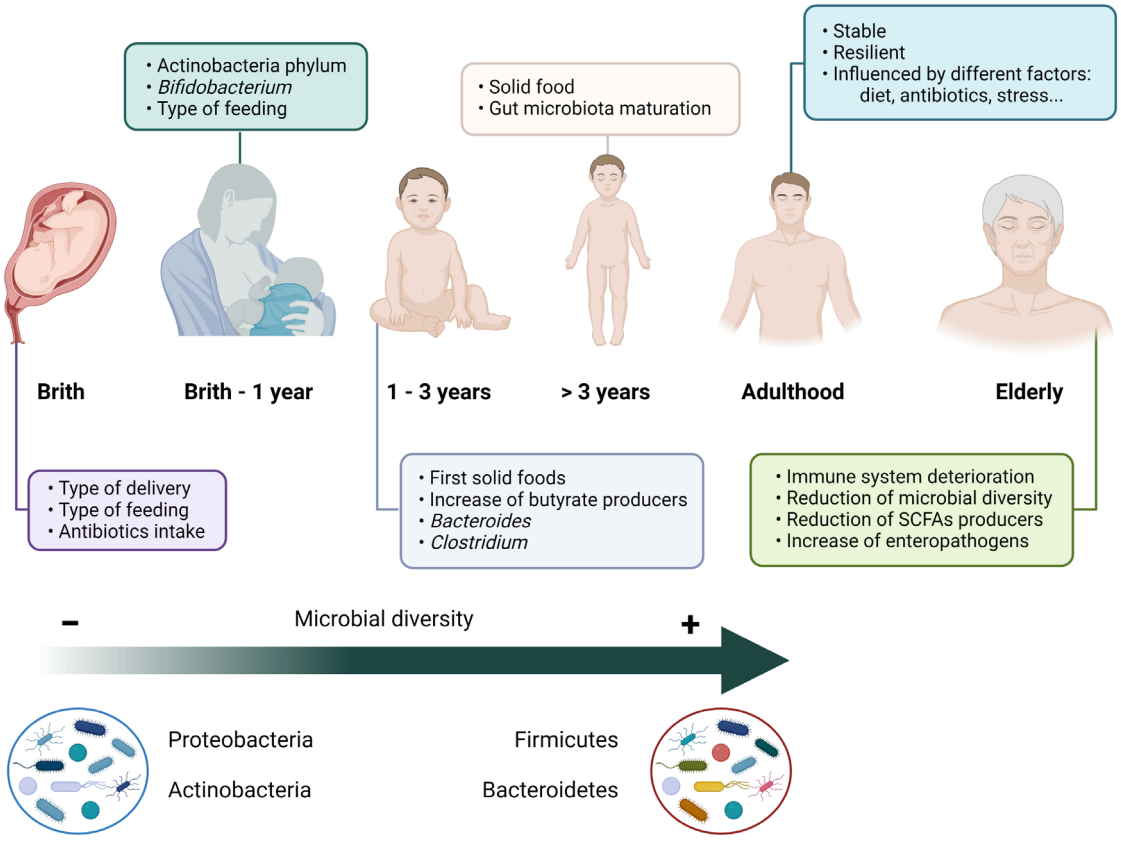


Figure 11. Evolution of gut microbiota composition along life. Gut microbiota composition evolves and is influenced by different factors along life. The main modulatory factors, as well as the main characteristics of the gut microbiota in the most important life periods are included.

On one hand, the gut microbiota profile evolves from the early childhood, when the most significant changes take place, to the adulthood and then to the elderly[105]. Some studies state the presence of microorganisms in the placenta or in the amniotic fluid,

suggesting that the microbiota colonization starts before birth[127,128]. Moreover, microorganisms have been detected also in the meconium of the neonates[129]. Nevertheless, latter investigations have shown that the bacteria identified in the placenta is not able to be distinguished from those contaminated from the surroundings[130], not supporting the idea of the placental microbiome[131,132]. Additionally, the ability to obtain germ-free animals from caesarean delivery is another contribution against it[132]. Hence, more studies are needed to clarify this debate. In any case, the first major exposure to microorganisms occurs during the birth[106]. The colonization of the gut microbiota of the neonate depends on different factors such as the type of delivery (vaginal or caesarean), the feeding (breastfeeding or formula feeding), the environment or the intake of antibiotics during the first weeks of life[100]. For example, a vaginal delivery promotes the colonization of microbes present in the mother's vagina, such as *Lactobacillus* or *Prevotella*, while caesarean delivery favors the establishment of microbiota from the mother's skin or the hospital environment, like *Staphylococcus*[116,133]. It is said that children born by caesarean have a less diverse gut microbiota and, although the consequences of this disruption in microbiota-transmission are unclear[106,116], an association with an increased risk of obesity has been established[134]. In general terms, the first microorganisms that colonize the gut after birth are aerobes and facultative anaerobes, predominating Proteobacteria and Actinobacteria phyla. Over time, the gut microbiota becomes anaerobic and a more diverse environment, including Firmicutes and Bacteroidetes phyla[108,134]. Additionally, the type of feeding significantly shapes the infant gut microbiota too, mainly due to the presence of human milk oligosaccharides[134]. In fact, approximately 25-30% of the infant gut bacteria belongs to the breast milk[129]. Related to that, Actinobacteria phylum dominates the gut microbiota between the first and the sixth month of life regardless of the type of delivery, mainly constituted by *Bididobacterium* genus[106]. However, the abundance of this genus starts to decrease when the infant introduces the first solid foods, approximately at six months of age, also period in which butyrate producers such as *Bacteroides* and *Clostridium* increases its concentration in order to catabolize the starch-based carbohydrates present in the diet[100,106,116]. Approximately at three years-old, the dietary patterns based on solid food increase the compositional and functionality of the gut microbiota[129]. It is also at this age when it is estimated that the infant gut microbiota gets established and resembles an adult-like one, although recent studies point out to the fact that gut microbiota maturation could take longer periods[135]. During the adulthood, the gut microbiota stays stable and resilient, although some factors such as diet, lifestyle,

stress, geographical location, or antibiotic intake are able to affect and influence it[105,112,116,134,135]. Moreover, Arumugam *et al.* proposed in 2011 to cluster the human gut microbiota of adults into three enterotypes based on the presence of an enrichment of *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) or *Ruminococcus* (enterotype 3) genera[136]. Thus, whereas enterotype 1 is mainly involved in proteolytic and saccharolytic activities, enterotype 2 is characterized by mucin glycoprotein degradation and enterotype 3 by mucin glycoprotein degradation and transportation of sugars[113]. However, different following studies suggest the presence of bias in this clustering, and there is still debate about its potential use since gut microbiota seems to be a continuum instead of three different clusters[113,116]. Finally, elderly promotes significant changes in the human gut microbiota mainly due to a deterioration of the immune system, the physiological changes along the digestive tube and the consequent reduction of food diversity[108,137]. In summary, there is a gradual specialization of the gut microbiota to digest the different substrates that are available in the gut along life[135].

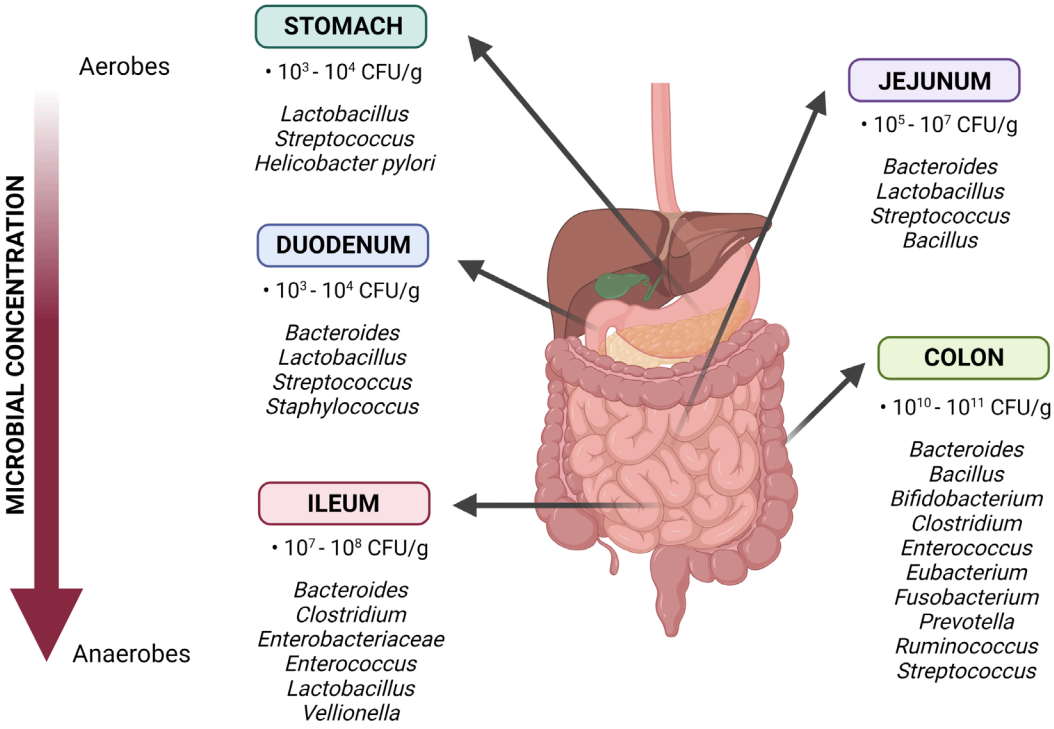


Figure 12. Gut microbiota composition along digestive tract. The main characteristic bacterial taxa of each region of the digestive tract together with the approximate bacterial concentration per gram of digestive content are included. CFU, colony-forming unit.

On the other hand, the different characteristics of each section of the gastrointestinal tract (pH, motility, oxygen availability…) confers the habitat for specific

bacteria, leading to a different gut microbiota composition along the digestive tube (**Figure 12**). Therefore, the stomach and the duodenum are the sections with the lowest gut microbiota concentration (10^3 – 10^4 colony-forming units (CFU) per gram) due to the acid pH and the presence of pancreatic secretions. The bacterial concentration increases in the jejunum and ileum, whereas the greatest values are reached in the colon, where an increase in pH, a decrease of the pancreatic secretions and a reduction in the intestinal motility favor the proliferation of the bacteria[105,122]. In fact, the colon is the place where the fermentation of undigested compounds and the water absorption take place due to its anaerobic environment[113]. Additionally, there is an enrichment in anaerobic bacteria along the digestive tube, so in the lower intestine dominates anaerobes such as *Bacteroides*, *Bifidobacterium* or *Fusobacterium*, whereas in the upper part are the aerobes and the facultative aerobes like *Lactobacillus* and *Enterobacterium* the predominant bacteria[116,138].

Overall, despite the fact that gut microbiota composition shares general patterns across the population, it is like our fingerprint: each individual has its own and different profile[116,119,133]. Moreover, it is worth mentioning that a healthy gut microbiota has been associated with high diversity and gene richness, although it has not been defined in terms of taxonomy. Additionally, the ability to resist different perturbations in the composition and return to a healthy pattern, called dynamic equilibrium, is another sign of health[119]. On the contrary, in the next chapter, the link between the gut microbiota and the development of obesity and non-alcoholic fatty liver disease will be discussed.

3.4. Relationship between Gut Microbiota and Metabolic Diseases

The relationship between gut microbiota and metabolic diseases, specifically obesity and NAFLD, has been deeply studied in the last twenty years. The research in the area began with experiments in animal models, as it is a feasible strategy to remove confounding variables that are present in humans, such as environment or genotype. In particular, germ-free (GF) mice were the 'gold-tool' to demonstrate the causality of the gut microbiota in the disease.

The first discovery that drew attention to the importance of gut microbiota in energy metabolism and, consequently, in the development of obesity was carried out by Bäckhed *et al.* in 2004. In this study, it was shown that the total body adiposity of GF

mice was 42% lower than the conventional mice from the same strain, even though the formers had a higher daily consumption of food. Moreover, GF mice that were colonized with caecal content from conventional mice experimented an increase in their body fat mass up to approximately 60%, fact that was accompanied by a reduction in the food consumption and an increase in hepatic triglycerides[139]. Two years later, Turnbaugh *et al*. performed a microbiota transplantation from lean or genetically induced obese mice to GF mice, showing in their results an increment of the body fat percentage in those mice colonized with caecal content from obese mice compared to the mice colonized with material from lean donors[140]. This finding was replicated by the same research group using a dietary mice model, in which obesity was induced with a Western diet (WD)[141]. These three studies were the first to demonstrate that the gut microbiota of obese mice has an increased ability for energy harvest from the diet, and consequently plays an important role in the development of the disease. In fact, the proposed mechanism to explain the ability of the gut microbiota promoting energy extraction from the diet is based on the absorption and fermentation of carbohydrates, process that increases the fatty acid synthesis and favors the triglyceride accumulation in the adipose tissue, contributing finally to obesity[142].

Despite the huge importance of these experiments in the field, their main limitation resided in the scarce extrapolation to humans, reason why the development of humanized mice, ergo, mice that have been transplanted with human microbiota, took place. In this sense, in the first study carried out with this strategy, the colonized mice showed an accurate and stable gut microbiota composition, which reproduced the human profile and was able to be transmitted to a second generation of mice without any loss in diversity. Additionally, mice that were colonized with caecal material from dietary-induced obese humanized mice had significantly more adiposity than those colonized with caecal microbiota from lean humanized mice, demonstrating again the role of gut microbiota in obesity[143]. Furthermore, another similar study conducted by Ridaura *et al*. reinforced the findings: female twins with a significant difference in their BMI were selected as donors of faecal material to perform a transplantation to GF mice. The results showed the ability of the microbiota derived from the obese sibling to colonize the gut of the GF mice and to reproduce in them the obesity phenotype, since they exhibited a higher total body weight and a higher percentage of fat mass compared to those that were colonized with microbiota from the lean sibling[144]. These experiments not only provided

a feasible tool to deepen the research of gut microbiota in health and disease, but also corroborated the previous and aforementioned findings related to obesity.

Once the role of the gut microbiota in the disease development is discussed, *what is known about its composition?* In 2005, the same research group that firstly demonstrated the link between gut microbiota and obesity published a study in which a comparison of the gut microbiota profile between obese and lean mice was performed, showing an increase in Firmicutes and a decrease in Bacteroidetes phylum, leading to a consequent increase in the known Firmicutes/Bacteroidetes ratio (F:B ratio) independently of the gender[145]. This finding was also observed in further studies, not only in mice but also in humans[140,141,143,146], considering during several years the F:B ratio as a fingerprint of obesity. However, different latter investigations have shown no differences in this ratio[114,147–149] and even an opposite trend[150], which could possibly be attributed to the multiple factors (region, lifestyle, race, age…) that influence the gut microbiota among different individuals. For those reasons, the F:B ratio is currently not considered as a good and trustable marker of obesity, even though there are authors that are still reporting it and that a reduction in the F:B ratio has been found as a sign of obesity remission[151,152]. Moreover, although *Lactobacillus* is considered a 'beneficial genus' due to its use as a probiotic, is consistently increased in obesity[134,151], fact that could be related to the different strains of the genus. Additionally, a reduction in *Oscillospira*, a butyrate producer, and *Bifidobacterium* genera has been reported[114,134,150], indicating that these two genera could have beneficial effects on metabolism. It is worthwhile to also mention a decrease in the strains *Akkermansia muciniphila*, able to degrade and stimulate mucins, and *Faecalibacterium prausnitzii*, a butyrate producer, in obesity[119,151,153]. Finally, regarding bacterial diversity, as the systematic review carried out by Crovesy *et al*. confirmed, the studies are still controversial, as some authors have found less diversity linked with obesity[154,155] while others have found the opposite trend[146]. Furthermore, it seems that gut microbiota richness is higher in patients with obesity[151]. Altogether, despite there are some taxa that are consistently related to obesity, there is not an established complete profile that could help us to elucidate the relationship between the composition and functionality of gut microbiota composition and obesity.

Considering the huge relationship between obesity and metabolism, the research on gut microbiota was extended to other metabolic disorders such as NAFLD. In fact, the contribution of the gut microbiota in NAFLD development was firstly carried out by Le

Roy *et al*. with an experiment in GF mice that were colonized with caecal microbiota from responder and non-responder mice to a high-fat diet (HFD)[156]. Additionally, different studies have tried to elucidate, as in obesity, a gut microbiota profile associated with NAFLD, although it is still under debate due to inconsistent results[157]. In general terms, it has been shown that patients with NAFLD have small intestinal bacterial overgrowth (SIBO)[158], as well as a higher abundance of Proteobacteria phylum and *Clostridium*, *Escherichia*, *Prevotella* and *Streptococcus* genera. Oppositely, lower levels of *Faecalibacterium*, *Ruminococcus* and *Coprococcus* have been detected[122,159]. In conclusion, as it happens with obesity, there is not a consensus about the gut microbiota profile associated with NAFLD, not only due to the huge differences among individuals based on age, race, lifestyle, or geography, but also because of the gut microbiota composition also changes with NAFLD stage. However, more studies are currently trying to identify general profiles that help to point out novel targets for the treatment of these diseases. Before knowing more about possible treatments for these pathologies, the mechanistic relationship between the gut microbiota and obesity and NAFLD should be addressed, reason of what, in the next chapter, the called 'gut-liver axis' is explained in detail.

3.5. Role of Gut Microbiota in the Gut-Liver Axis

As it was previously explained, the gut microbiota is undoubtedly implicated in the development of obesity and NAFLD. However, in order to fully understand this bidirectional interaction between gut microbiota and disease, an accurate mechanistic and molecular explanation is needed. In this chapter, we will deepen in the concept of gut barrier, its composition and integrity, clarifying how it can be altered, and justifying how the different metabolites derived from the gut microbiota can promote obesity and NAFLD.

Firstly, gut and liver are anatomically communicated by the portal vein, so approximately 70% of the blood that the liver receives comes from the gut, whereas the gut gets hepatic secretions through the biliary tree. This narrow relationship between these two organs is called 'gut-liver axis' and constitutes the relevant point to understand the link between gut microbiota and liver diseases[56].

In a healthy status, the gut is provided with a barrier whose main function is the the maintenance of the physiological homeostasis of the host, avoiding that pathogens or

toxic metabolites derived from the gut microbiota translocate and reach the systemic circulation[160]. As shown in **Figure 13A**, this gut barrier is constituted by the mucus layer, the epithelium cells, the lamina propria and the different immune cells involved. The mucus is the first line of defense and is a double layer constituted by mucin glycoproteins and glycans with hydrophobic and surfactant properties. These substances are synthetized by goblet cells in order not only to avoid the attachment of microorganisms to the epithelium and their posterior translocation, but also to lubricate and to allow the absorption of beneficial compounds[161]. The thickness of this barrier is different along the gut, being greater in the ileum and colon since it is there where the microbiota concentration is higher. The outer layer is the one that is in direct contact with the gut microbiota, so many bacteria are attached there in order to prevent being expelled by peristalsis[162]. Contrarily, the inner layer is more sterile and constitutes a barrier that separates the microbiota from the epithelium[113]. Gut microbiota determines the composition of the mucus layer, which also constitutes a source of nutrients such as carbohydrates or peptides for the bacteria[162]. Secondly, a monolayer of epithelial cells is located just below the mucus, constituting the second barrier of the gut but, at the same time, allowing the absorption of nutrients[163]. These cells are kept joined due to the presence of three intercellular type of junctions: tight junctions (TJs), adherens junctions and desmosomes. Therefore, TJs, mainly claudins, occludins and junctional adhesion molecules (JAMs), are transmembrane proteins located in the apical plane that are involved in the control of permeability and vesicle transport. Additionally, scaffold proteins like zonula occludens (ZO) are in charge of maintaining the link of TJs with the cytoskeleton of the cells[161]. This epithelial barrier is not only physical since the cells also synthetize antimicrobial peptides to avoid bacteria translocation. Following by this epithelium, it can be found the lamina propria, constituted by plasma cells that also protect the gut, and the gut vascular barrier (GVB), which prevents the entrance of the bacteria to the portal and systemic circulation[162]. In addition, the presence of different immune cells such as T cells, B cells or macrophages, not only in the epithelium but also in the lamina propria contributes to the barrier reinforcement. In this sense, Paneth cells, located in the base of the gut crypts, secrete antimicrobial peptides on the epithelial surface[164]. Altogether, these cells are involved in the induction of tolerance to microbial-derived compounds and nutrients but, at the same time, they constitute another line of defense of the host[161]. To sum up, the gut barrier is constituted by structural, immune, and

biochemical mediators that protect the host from the possible damage of antigens, microbial-derived compounds, and microorganisms.

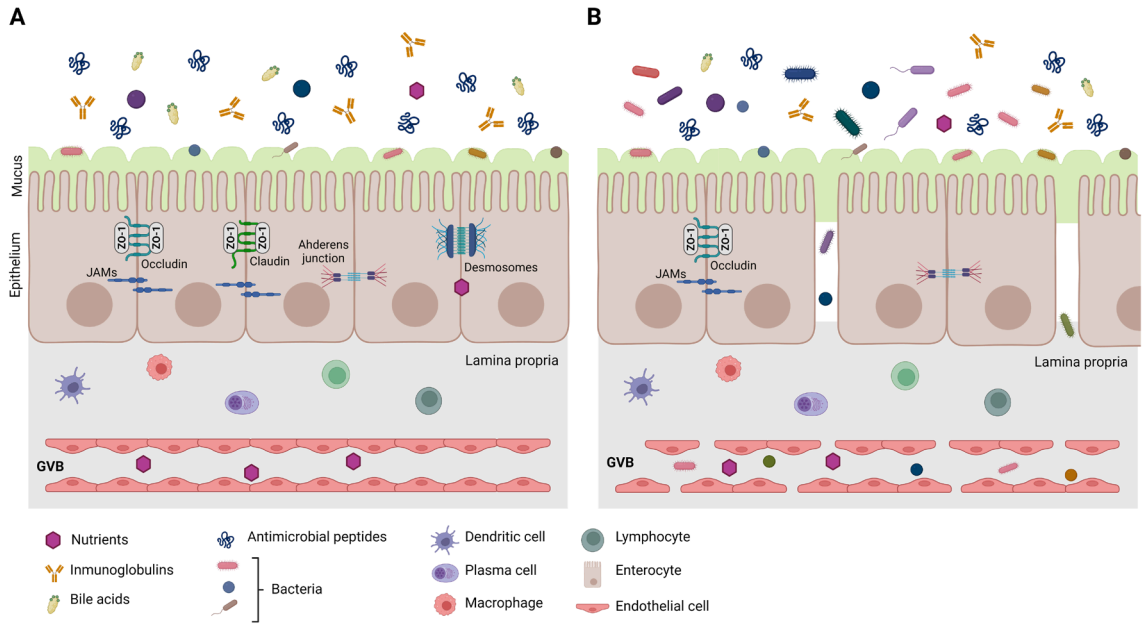


Figure 13. Gut barrier structure. A. Structure and main components of the gut barrier in a healthy status: mucus layer, epithelium cells joined by intracellular junctions (tight junctions, adherens junctions and desmosomes), the lamina propria, the gut vascular barrier and the presence of different immune cells. **B.** Structure and main components of the gut barrier in a disease-related status. Gut microbiota alteration and disease-related status modify mucus layer, epithelium cells and junctions, gut vascular barrier and, consequently, the status of the whole gut barrier. GVB, gut vascular barrier; JAMs, junctional adhesion molecules; ZO-1, zonula occludens 1.

3.5.1. Alteration of the gut barrier and endotoxemia

In 2007, Cani *et al.* demonstrated for the first time that the plasma concentration of the lipopolysaccharide (LPS), an element of the outer wall of the Gram-negative bacteria, was higher in high-fat diet-fed mice compared to control mice, fact that led to hepatic inflammation, insulin resistance and NAFLD development[165]. Based on these results, the authors established the concept 'metabolic endotoxemia' to refer to that status and, since then, different studies have been focused on the gut barrier and the endotoxemia as possible contributors to obesity and NAFLD diseases[166].

Now, it is known that an alteration in the composition of the gut microbiota contributes to a disruption of the gut barrier integrity, which leads to the translocation of different microbial products such as LPS or endotoxins, and even microorganisms, to the liver and to the systemic circulation, thereby promoting damage and an inflammatory status[160] (**Figure 13B**). In this sense, the gut microbiota composition, as it was mentioned

before, regulates the thickness of the mucus layer, and also regulates the tight junction proteins, so an altered microbial profile is able to disrupt the gut barrier integrity[142]. Once the microbial products reach the liver through the portal circulation, they are recognized as pathogen associated molecular patterns (PAMPS) by different pattern recognition receptors (PRR), mainly Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs), which activate a proinflammatory signaling pathway with the consequent release of proinflammatory cytokines and the development of an inflammatory status[167]. Moreover, these proinflammatory cytokines are also able to alter the tight junctions and to increase gut barrier permeability, contributing to a loop that increases inflammation, steatosis and insulin resistance[164].

The inflammatory response takes place in two different ways (**Figure 14**). On one hand, through the TLRs, transmembrane proteins located in cells such as macrophages or dendritic cells that recognize different PAMPs (LPS or bacterial DNA, among others) and danger-associated molecular pattern molecules (DAMPs)[164]. Thus, different microbial antigens are recognized by different types of TLRs: lipopolysaccharide is recognized by TLR2, peptidoglycan by TLR4, flagellin by TLR5 and bacterial DNA by TLR9, among others[167]. The activation of these receptors leads to the induction of the NF-κB pathway, the release of proinflammatory cytokines, and the contribution to the inflammatory status. On the other hand, the recognition of PAMPs and DAMPs by NLRPs produces the activation of inflammasomes, as NLRP3 inflammasome, which also leads to an inflammatory status[142].

In addition, it is worth it to carefully explain the recognition of the LPS, as it is one of the most potent activators of the inflammatory response[164]. In general terms, the alteration of the gut microbiota during or as a prior state of a disease is usually related to an increase in pathogens and the consequent increase in LPS levels[168]. The lipid A of the LPS is recognized firstly by the LPS-binding protein (LBP), which is present in the blood, constituting the LPS-LBP complex. Next, this complex interacts with CD14, an adapter protein that lets LPS be recognized by TLR4, located in the membrane of hepatocytes or Kupffer cells. The activation of TLR4 induces, as it was previously reported, the NF-κB pathway and the release of proinflammatory mediators and cytokines such as TNF, IL-18, or IL-6[169].

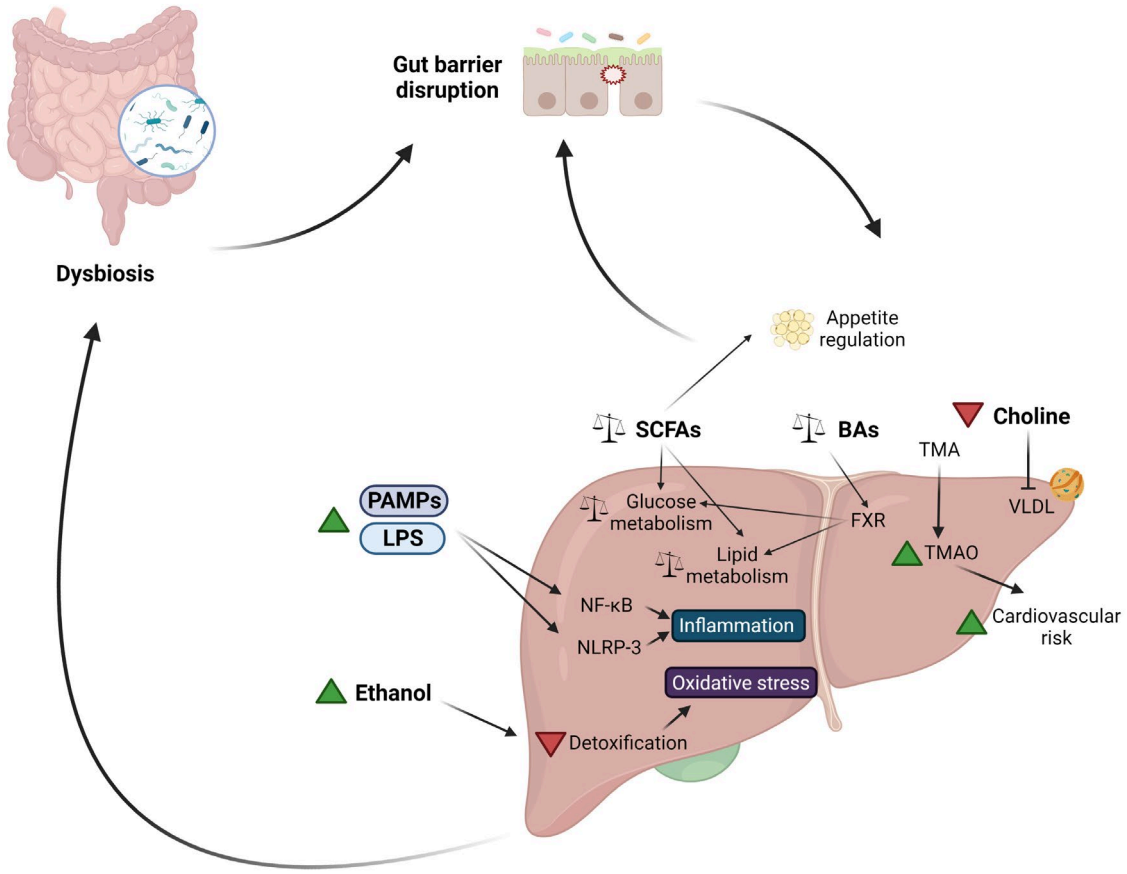


Figure 14. Gut-liver axis. Main microbial-derived metabolites and mechanisms involved in NAFLD pathogenesis. BAs, bile acids; FXR, farnesoid X receptor; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; NLRP3, NOD-like receptor family pyrin domain containing 3; PAMPs, pathogen-associated molecular patterns; SCFAs, short-chain fatty acids; TMA, trimethylamine; TMAO, trimethylamine N-oxide; VLDL very low-density lipoprotein.

Based on the aforementioned information and considering that the alteration of the gut microbiota is a contributor factor of the development of obesity and NAFLD, different studies have demonstrated in animal models and in humans the presence of metabolic endotoxemia and gut barrier disruption on these diseases[170–175]. Additionally, SIBO, as it was previously mentioned, is one of the characteristics of NAFLD patients and it has been suggested that it could also constitute an important factor in the development and aggravation of the disease, since these microorganisms could alter the gut barrier and damage the liver[169]. In this sense, it has been found that patients with NAFLD have SIBO and increased gut permeability, both factors that positively correlated with the severity of the disease[176,177]. Moreover, another study performed in rats demonstrated that LPS is able to induce the expression of genes related to lipid accumulation in the liver, favoring NAFLD development[178]. Altogether, different studies

sustain the important role of metabolic endotoxemia and gut barrier disruption in the development of obesity and NAFLD.

Lastly, the alteration in the gut microbiota composition undoubtedly modifies its function and, consequently, the produced microbial metabolites, which are involved in the development of obesity and NAFLD[160]. The main metabolites derived from the gut microbiota and their relationship with these diseases are summarized in **Figure 14** and explained below.

3.5.2. Short-chain fatty acids (SCFAs)

Short-chain fatty acids are saturated fatty acids that are produced by the gut microbiota from the fermentation of non-digestible carbohydrates and proteins[163]. The main species are acetate, propionate and butyrate, even though minor amounts of others such as lactate or branched chain fatty acids are also synthetized[179]. The concentration of these three main SCFAs depends on the substrate, the gastrointestinal motility, and the specific composition of the gut microbiota. They can be found in the colon, where they are mainly produced, in a general molar ratio of 60:20:20[163]. It is estimated that approximately 5-10% of the energy demanded in a normal status is supplied by SCFAs[180] and, while butyrate is mainly metabolized in the colon, acetate and propionate are able to reach the liver through the portal vein[181]. The important role of acetate, propionate and butyrate in physiological homeostasis has been deeply investigated, as these compounds affect gut integrity, glucose homeostasis, appetite regulation, lipid metabolism and inflammation. Butyrate, the main source of energy of the colonocytes, is the main specie involved in the maintenance of gut integrity, as regulates tight junction proteins, enhances the barrier function and is able to diminish the translocation of LPS[182]. Regarding glucose homeostasis, propionate is involved in hepatic gluconeogenesis, while acetate participates in lipogenesis and cholesterol synthesis in the liver, both processes that can be inhibited by propionate[111]. Therefore, the hepatic ratio propionate:acetate could be important in the lipid storage[179]. SCFAs also play an important role in the modulation of the inflammatory and immune status in the gut: butyrate and propionate hamper the production and release of proinflammatory cytokines, while acetate regulates intestinal inflammation[183]. Regarding their receptors and its role in appetite regulation, SCFAs activate the G-protein-coupled receptors (GPRs) GPR41 and GPR43, renamed as free fatty acid receptors (FFARs) FFAR2 and FFAR3[163], that are located in the endocrine L cells of the

gut. This binding promotes the stimulation and release of the peptide YY (PYY) and incretin GLP-1, diminishing the speed of gastric emptying and, consequently, promoting satiety. Moreover, the secretion of GLP-1 regulates the activation of genes involved in hepatic fatty acid oxidation and insulin sensitivity[160,180]. Recently, other GPCRs such as GPR109A, Olfr78 and Olfr558 have been also identified as SCFAs receptors[183].

The role of SCFAs in obesity and NAFLD has been deeply investigated. On one hand, due to the functions of SCFAs in the appetite regulation and due to the ability of these molecules to stimulate leptin secretion, it is easy to underneath that they undoubtedly are important in obesity. However, if SCFAs are or are not beneficial in the disease is still unknown since a lot of controversial results have been reported. To begin with, based on the aforementioned SCFAs functions, they seem to have an anti-obesogenic effect by reducing appetite and promoting satiety, as well as by the ability to suppress *de novo* lipogenesis and triglyceride accumulation. Additionally, an induction of browning of white adipose tissue has been also reported as a SCFAs function[181]. In humans, lower levels of propionate and butyrate in faeces have been identified in children with obesity and overweight[184], and epidemiological studies have stated an inverse correlation between the intake of dietary fibers and the body weight[185]. In this sense, different animal and human studies have demonstrated the beneficial effects of SCFAs in obesity, as the supplementation with these species is able to reduce body weight and adiposity[185–188]. Contrarily, other studies have reported higher faecal concentrations of SCFAs in patients with obesity compared to control individuals[140,150,155,189,190], probably due to the ability of these molecules to harvest more energy from the diet. In fact, genetically induced obese mice have higher caecal SCFAs levels and lower faecal energy content compared to lean mice[185], demonstrating the role of SCFAs in energy harvest. However, no human data is available regarding this fact. Moreover, during obesity, the activation of GPRs by SCFAs might be attenuated, reducing the satiety signal, and promoting energy harvesting[190]. To sum up, SCFAs have an important role in obesity development, with paradoxical effects that need to be further studied.

On the other hand, the role of SCFAs in NAFLD seems to be protective due to their ability to maintain the gut barrier integrity, to reduce inflammation, and to regulate fat accumulation in the liver and in adipose tissue[191], even though, as occurred with obesity, the debate is still open. In this sense, the administration of different SCFAs in mice reduced hepatic steatosis and improved insulin sensitivity[192,193]. Moreover, a

reduction in SCFAs levels in non-obese NAFLD patients compared with healthy subjects has been reported[194]. Different authors have identified high levels of acetate, butyrate and propionate in patients with different stages of NAFLD[167]. In fact, whereas high acetate faecal concentration has been detected in patients with advanced fibrosis, elevated butyrate and propionate faecal levels have been identified in patients with moderate NAFLD[180], differences that could be attributed to various factors such as age, ethnicity, diet, among others. All in all, similar to obesity, SCFAs might play a paradoxical role in NAFLD development, an issue that should be addressed with further studies. To this must be added the narrow relationship between these two pathologies, which complicates the comprehension of the link among SCFAs and the pathophysiology of obesity and NAFLD.

3.5.3. Bile acids

Bile acids (BAs) are amphipathic molecules synthetized from cholesterol in the liver, stored in the gallbladder and released during the feeding into the gut, where facilitates the transport, absorption and excretion of lipids and lipophilic vitamins by micelles formation[116]. However, the functions of BAs are not only limited to the nutritional field, since different roles in immune system, glucose homeostasis and lipid homeostasis have been also identified, reason of which these molecules are important in the gut-liver axis[195].

The BAs pool is constituted by primary and secondary species. In humans, the main primary bile acids are cholic acid (CA) and chenodeoxycholic acid (CDCA), which are synthetized by the hepatocytes and subsequently conjugated to taurine or glycine[196]. The synthesis of primary BAs comprises two different multi-step pathways, as it is shown in **Figure 15**: the classical pathway, in which cholesterol 7α-hydroxylase (CYP7A1) and cholesterol 8β-hydroxylase (CYP8B1) enzymes are involved, with the latter being the rate-limiting enzyme; or the alternative one, in which sterol 27-hydroxylase (CYP27A1) has the leading role. Additionally, the multidrug resistance protein 3 (MDR3, or MDR2 in mice) participates in the synthesis of phospholipids to the bile. Following the synthesis, they are conjugated to glycine or taurine through a reaction where enzymes such as bile acid-CoA: amino acid N-acyltransferase (BAAT) are implicated. After that, primary species are transported mainly by the bile salt export pump (BSEP) and the multidrug resistance-associated protein 2 (MRP2) through the canalicular membrane and stored in the gallbladder[197]. During a meal, these conjugated species are released into the

duodenum to exert the aforementioned functions: facilitating the transport and absorption of lipophilic nutrients. Reached the gut, the primary BAs constitute the substrate of different microbial enzymes that transform them by different steps such as deconjugation, dihydroxylation or epimerization into secondary BAs: deoxycholic acid (DCA), derived from CA; and lithocholic acid (LCA) and ursodeoxycholic acid (UDCA), derived from CDCA[116]. The deconjugation step made mainly by the bile salt hydrolase (BSH) enzyme presented in many bacteria constitutes a mechanism of reduction of the toxicity of the BAs, as well as a way to confer a source of different useful compounds such as nitrogen[111]. Approximately, 95% of the pool of BAs are reabsorbed in the distal ileum and transported back to the liver, where they are absorbed by sodium taurocholate cotransporting polypeptide (NTCP) and organic anion transporting polypeptide (OATP). In the liver, secondary BAs are processed and conjugated with glycine and taurine in order to reduce their toxicity[111]. The remaining 5% is excreted through the faeces, equivalent quantity to the amount of BAs synthetized *de novo* by the liver. This process, in which the majority of BAs are recycled by reabsorption through the hepatic portal circulation, is called the enterohepatic circulation[196].

It is worth mentioning that the pool of BAs in rodents is relatively different from the human one, as the main primary BAs are not only CA and CDA, but also muricholic acids (MCA) αMCA and βMCA, which are synthetized from CDA (**Figure 15**)[195]. Additionally, whereas in humans primary BAs are conjugated both with glycine and taurine, in mice and rats the conjugation is mainly with taurine. Another important difference resides in the synthesis pathways: while in humans the classical pathway is the main one, in rodents both classical and alternative pathways have an important role in BAs formation. Besides, mice use both BSEP and MRP2 transporters to efflux BAs into the bile, while humans use mainly the former one[197]. Lastly, the secondary BAs in rodents are, in addition to DCA, LCA and UDCA, murideoxycholic acid (MDC), hyodeoxycholic acid (HDCA) and ω-muricholic acid (ωMCA) (**Figure 15**)[198].

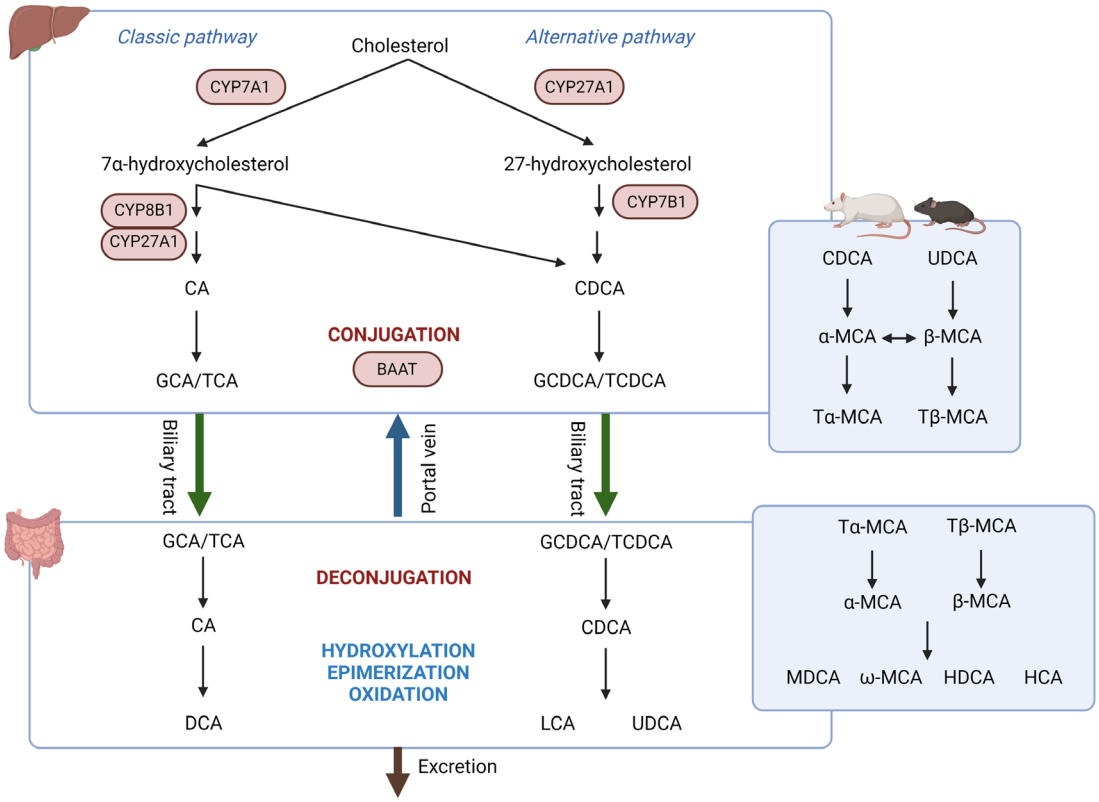


Figure 15. Bile acid metabolism: synthesis and enterohepatic circulation. Primary bile acid synthesis takes place in the liver through the classical and the alternative pathways. After its conjugation with glycine or taurine, and during a meal, bile acids are transported through biliary tract to the gut, where are deconjugated and metabolized into secondary bile acids by the gut microbiota. The majority of the bile acids are recirculated through the portal vein and a minor part of them are excreted through faeces. The main differences in bile acid metabolism in rodents are included in the blue boxes and the main enzymes are highlighted with red boxes. CA, cholic acid; CDCA, chenodeoxycholic acid; CYP27A1, sterol 27-hydroxylase; CYP7A1, cholesterol 7α-hydroxylase; CYP7B1, cholesterol 7β-hydroxylase; CYP8B1, cholesterol 7β-hydroxylase; DCA, deoxycholic acid; GCA, glycocholic acid; GCDCA, glycodeoxycholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LCA, litocholic acid; MCA, muricholic acid; MDCA, murideoxycholic acid; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; UDCA, ursodeoxycholic acid.

As it was mentioned before, BAs are not only involved in the absorption of lipophilic compounds in the gut, as they are also signaling molecules that regulate different pathways such as lipids and BAs metabolism or inflammation, through their binding with farnesoid X receptor (FXR), Takeda G protein-coupled receptor 5 (TGR5), also known as G protein-coupled bile acids receptor 1 (GBPAR1), or pregnane X receptor (PXR)[115]. On one hand, the regulation of BA metabolism is mainly performed by the activation or inhibition of FXR, as the different species of BAs have antagonist or agonist effects on this receptor. For example, while primary CDCA stimulates FXR, secondary DCA inhibits it[199]. The BAs able to stimulate FXR in the gut provoke the induction and

secretion into the portal circulation of the human protein fibroblast growth factor 19 (FGF19) (FGF15 in mice). This molecule inhibits the BA synthesis in the liver through the suppression of CYP7A1 and CYP8B1, playing an important role in BA homeostasis[197]. During enterohepatic circulation, the BAs that are reabsorbed in the liver also stimulate hepatic FXR, resulting in the expression of the small heterodimer partner (SHP), which also inhibits BA synthesis[196]. Additionally, FXR is involved in glucose metabolism, as increases insulin sensitivity, inhibits hepatic gluconeogenesis through SHP, as well as favors the glucose uptake in adipose tissue through FGF19 activation. Regarding lipid metabolism, FXR hampers lipogenesis and activates fatty acid oxidation[191,199].

On the other hand, the intestinal activation of TGR5 by the BAs produces the secretion of PYY, GLP-1 and GLP-2, three appetite-related hormones, which leads to a reduction in the intestinal emptying and in the food intake[200]. Besides, this interaction stimulates energy expenditure through TGR5 activation both in brown adipose tissue and skeletal muscle, as well as regulates insulin metabolism and glucose metabolism[196,201]. TGR5 activation also modulates immune response and inflammatory status, as it is able to inhibit the expression of different proinflammatory molecules such as IL-6, TNF, or cyclooxiygenase-2 (COX2), as well as to inhibit NLRP3 inflammasome activation, which altogether maintains the gut barrier integrity and function[191].

In addition, there is a bidirectional relationship between gut microbiota and BAs: gut microbiota composition modulates not only the BAs pool, but also their structure and properties, while BAs pool has antimicrobial activities, such as the detergent effect of DCA on bacterial membranes, able to shape the gut microbiota community[111]. Moreover, BAs also regulate gut microbiota composition through the stimulation of FXR and TGR5, which leads to the activation of different antimicrobial pathways that are involved in the innate immune system and protect the gut barrier[118,198].

As an altered gut microbiota composition is a contributor and a characteristic factor of obesity and NAFLD development, and considering that the narrow link between gut microbiota and BA metabolism has an essential role in physiological functions, the study of BAs in these diseases could point out not only to elucidate their pathogenesis, but also to consider different therapeutic options. Therefore, the alteration in gut microbiota composition could lead to an imbalance in the BAs pool, fact that directly delivers in an impaired BA-related signaling[196] that could be involved in NAFLD and

obesity development. Numerous studies have demonstrated an altered BA profile in metabolic disorders such as obesity, NAFLD and T2DM, although there is still no consensus about the disease-related specific pattern[202]. Briefly, patients with obesity that underwent bariatric surgery had higher levels of FGF19, measure that correlated with an improvement in the disease[203]. Additionally, bariatric surgery also increased postprandial GLP-1 and PYY levels, fact that could be involved in the process of weight loss after the surgery[118]. Regarding NAFLD, high levels of secondary BAs have been associated with the disease. Moreover, an increase in those BAs with antagonist activity on FXR, as well as an increase in secondary BA metabolism have been detected in NAFLD patients. For that reason, a current field of research is focused on the potential role of FXR agonists in NAFLD treatment[204], although confounding results have also been pointed out probably due to the specific functionality of the receptor depending on the tissue[195].

In conclusion, although is not completely clear the specific BA pattern associated with these diseases, no doubts exist about the essential role of BA metabolism in their pathogenesis, being necessary more studies to clear up these relationships.

3.5.4. Ethanol

The gut microbiota profile that has been associated with NAFLD, as it was explained before, is enriched in some genera such as *Escherichia*, a group of bacteria characterized by its ability to produce ethanol[180]. Ethanol has been identified as a contributor to the alteration of the gut barrier integrity, favoring an increase in its permeability which leads to hepatic inflammation[160]. Based on that, it has been hypothesized that NAFLD patients have higher levels of ethanol compared to healthy humans due to their gut microbiota composition, fact that has been demonstrated in different studies[205–207]. Moreover, the hepatic detoxification pathways for this compound are weakened in NAFLD patients, leading to a higher production of ROS and the consequent oxidative stress, inflammation, and the aggravation of the disease[180]. Additionally, some gut bacteria transform the ethanol in acetaldehyde, molecule that disrupts the tight junctions of the gut barrier[167]. Regarding obesity itself, ethanol has not been identified as an important factor that exacerbates the disease.

3.5.5. Choline metabolism

Choline is a macronutrient obtained from different sources such as eggs or cheese that, as it was mentioned in the gut microbiota functions chapter, has important functions regarding lipid metabolism. Apart from its role in the maintenance of the structure and function of cell membranes' phospholipids, choline is involved in the production of VLDL in the liver[180]. Therefore, a deficiency in this macronutrient leads to a decrease in the VLDL production and, consequently, to an accumulation of hepatic triglycerides, fact that has been associated with NAFLD development[167]. Such is the importance of this process that choline-deficient diets have been widely used to induce NAFLD in animal models. In addition, choline is transformed into trimethylamine (TMA) by the gut microbiota, compound that is oxidized in the liver producing trimethylamine N-oxide (TMAO). TMAO levels have been related to NAFLD and obesity development and have been correlated with the severity of the diseases and with cardiovascular risk[180].

3.6. The Microbiota-Mitochondria Crosstalk and its Relevance in Obesity and NAFLD Diseases

Mitochondria, double-membrane organelles that have evolved from α-Proteobacteria, are involved in many functions such as calcium homeostasis, regulation of innate immune response, generation of free radicals or processes related to cell survival or death. However, the essential role of mitochondria resides in the production of adenosine triphosphate (ATP), the energetic coin of our organism[68]. In fact, approximately the 90% of the cellular energy is generated by these organelles in form of ATP production[208].

Mitochondria are found in the cytoplasm of our cells with a specific structure that allows them to carry out their functions: an outer membrane, which is permeable and delimits the intermembrane space; and the inner membrane, which bounds the mitochondrial matrix, is much less permeable and forms the mitochondrial cristae due to its high folding[71] (**Figure 16A**). The mitochondrial DNA (mtDNA) is located in the mitochondrial matrix, is packaged as circular double-strand molecules, and is only maternally transmitted[74,209]. This genetic material just encodes thirteen polypeptides, so the other proteins needed to carry on their functions, which are encoded by nuclear DNA, are synthetized in the cytoplasm and transported to the mitochondria through a regulated process[68].

Regarding the production of ATP, the inner membrane contains the electron transport chain (ETC), which is constituted by five complexes (I, II, II, IV and V). Thus, the coenzymes NADH and $FADH_2$, produced during the tricarboxylic acid cycle (TCA), donate their electrons that are accepted and transferred along the ETC, from complex I or II to complex III, complex IV and finally complex V. This transference of electrons produces a proton pumping across the ETC, generating an electrochemical gradient that generates ATP[208,210] (**Figure 16B**). During this whole process, called oxidative phosphorylation (OXPHOS), some electrons leak out and interact with oxygen to produce ROS, harmful for the mitochondria and cells, reason of which different defense and quality control mechanisms are used by the organism to alleviate the consequent-generated oxidative stress. Therefore, even mitochondria are the main ROS producer, it counts with endogenous enzymatic and non-enzymatic antioxidant pathways to counteract the oxidative stress[74]. However, these mechanisms could be overload, producing an imbalance between the antioxidant mechanisms and the production of ROS that leads to the damage of mitochondrial and nuclear DNA, mitochondrial dysfunction and cell harm[71,210].

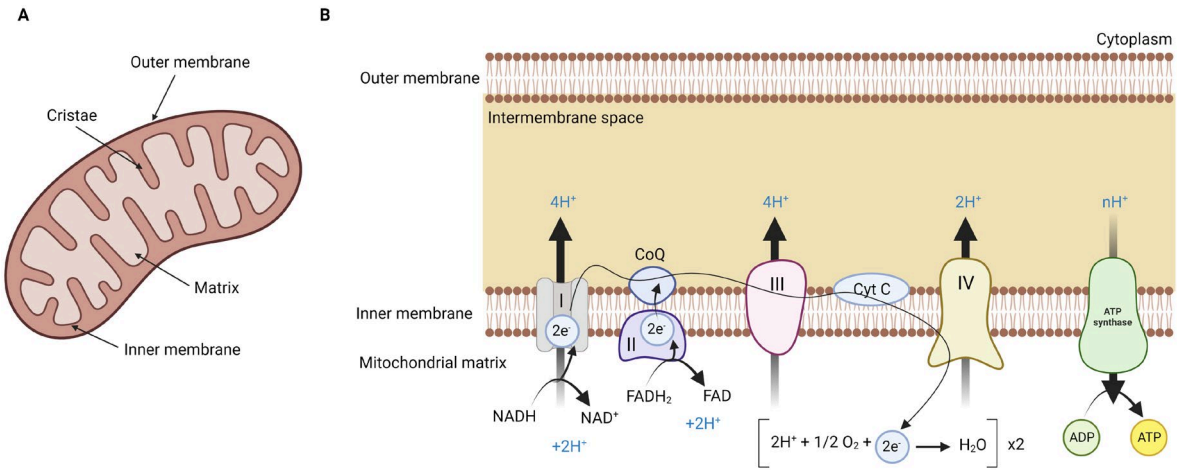


Figure 16. Mitochondria structure and function. A. Mitochondria structure. **B.** Mitochondrial electron transport chain (ETC). ATP; adenosine triphosphate; ADP, adenosine diphosphate; CoQ, coenzyme Q; Cyt C, cytochrome C; $FADH_2$, flavin adenine dinucleotide; NADH, nicotinamide adenine dinucleotide.

It has been several years since the role of mitochondria in disease was suggested, not only due to mutations in mitochondrial or nuclear genes, which lead to mitochondrial-related pathologies, but also because of mitochondrial dysfunction has been related with cancer, neurodegenerative and metabolic diseases[208,211]. In fact, as it was mentioned in previous chapters, oxidative stress and mitochondrial dysfunction play a key role in

obesity and NAFLD development. However, recent studies have pointed out that this mitochondrial dysfunction is also involved in intestinal diseases, hinting the existence of a mitochondria-microbiota crosstalk[212]. The gut epithelium needs high amounts of energy, as it is constantly being renewed, reason of which mitochondria play an essential role in its homeostasis[210]. Thus, the dysfunction in these organelles affects the self-renewal of the intestinal cells and also their differentiation[209]. In this sense, different gut microbiota metabolites such as SCFAs, nitric-oxide species or hydrogen sulfide can modulate and interfere with mitochondrial biogenesis, function, and metabolism[213]. For example, secondary BAs regulate different transcription factors involved in lipid metabolism which control mitochondrial function. Additionally, butyrate, one of the main SCFAs, also affects the mitochondrial metabolism in different ways, such as modulating peroxisome proliferator-activated receptor-gamma coactivator (PGC) 1 alpha, one of the main regulators of OXPHOS process, or enhancing the expression of antioxidant regulators with the aim of reducing ROS production[212,213]. Bacteria could also affect mitochondrial dynamics, regulating processes such as autophagy, fission or fusion. Based on that, an alteration in the gut microbiota profile directly affects intestinal energetic homeostasis, not only through the activity of the bacteria releasing different toxins and LPS but also through the derived metabolites, that altogether alters mitochondrial status. In the other way, changes in the mitochondrial metabolism also affect gut microbiota composition since a significant change in the gut environment takes place[212].

To sum up, the narrow relationship between gut microbiota, gut microbiota metabolites and mitochondria appears to be significantly involved in NAFLD and obesity development, being necessary to deepen in the specific mechanisms in order to explore new therapeutic alternatives.

In the last years, methylation-controlled J protein (MCJ) has emerged as a promising therapeutic study field. MCJ, also known as DnaJC15, is a member of the DnaJC chaperones family and its main function is acting as a negative regulator of the ETC[214]. This transmembrane protein constituted by only 147 amino acids was firstly identified in human ovarian cancer cells[215,216], but now it is known to be expressed also in immune cells such as CD8 or macrophages[214], and in tissues with a high mitochondrial metabolism, such as liver or kidney[217]. MCJ protein is located in the inner membrane of the mitochondria, where negatively regulates the complex I of the ETC and diminishes ATP production[218]. Specifically, it interferes with the formation of the super complexes

of the ETC, whose main role is facilitating the transference of electrons through the ETC and, thereby, minimizing the risk of ROS production[214] (**Figure 17**). Moreover, it has been shown that MCJ is nonessential in normal physiological conditions and its loss increases not only the activity of complex I but also the ATP production without augmenting the ROS production[214,216].

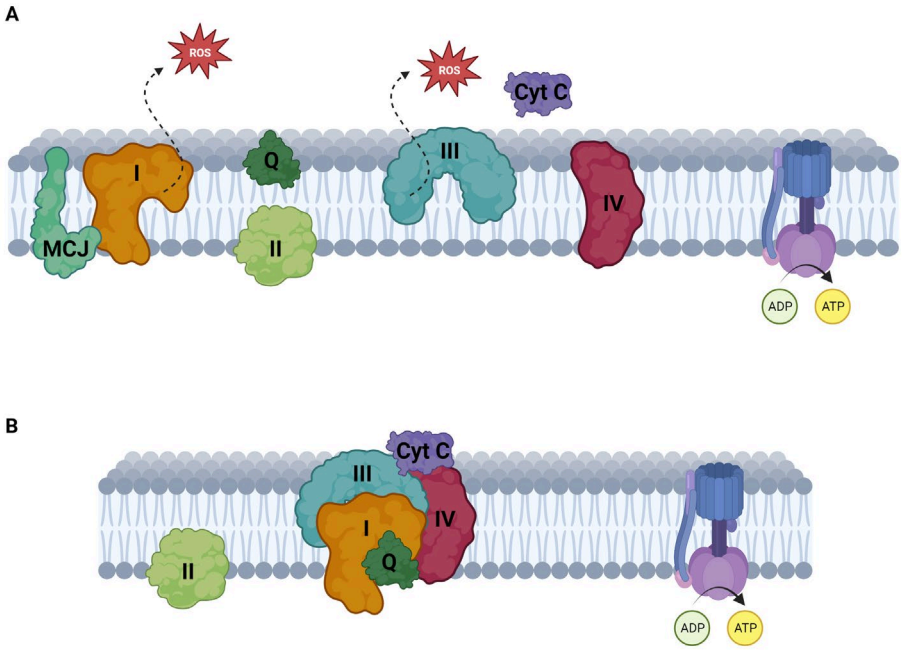


Figure 17. Methylation-controlled J protein (MCJ) function in the mitochondrial electron transport chain. A. Representation of the function of MCJ protein in the electron transport chain, inhibiting the complex I activity. **B.** Representation of the MCJ deficiency in the electron transport chain function. ATP; adenosine triphosphate; ADP, adenosine diphosphate; Cyt C, cytochrome C; ROS, reactive oxygen species; Q, coenzyme Q.

Different studies have demonstrated the role of MCJ in liver metabolism. Hatle *et al*. firstly described that MCJ deficiency is able to counteract the accumulation of hepatic lipids in mice[214] and, a few years later, Barbier-Torres *et al*. showed that this protein participates in the liver injury induced by acetaminophen. In fact, MCJ deficiency and/or MCJ silencing were able to prevent hepatic oxidative stress and hepatic damage, as well as to increase the regenerative capacity of the liver after acetaminophen administration[217]. As it was not enough, MCJ has been also reported to regulate the development of cholestatic liver diseases[219]. Moreover, the carried research on NAFLD field is also promising. MCJ knock-out mice have shown to be resistant to steatohepatitis development and, additionally, hepatic MCJ silencing improves steatosis and fibrosis in different mice models[220]. These results point out the MCJ silencing as a possible

therapeutic field, something that is supported by the high levels of MCJ protein that have been detected in the liver of patients with NASH[220]. However, considering the above-mentioned microbiota-mitochondria crosstalk as well as the gut-liver axis, information about the link between the intestine, the microbiota and MCJ protein in NAFLD is still scarce. The available information only covers intestinal diseases, and, specifically, ulcerative colitis. During an acute state of this disease, MCJ seems to play a protective role in mice, as its deficiency led to a more severe disease characterized by an alteration in the gut microbiota composition, an increased in gut permeability and an enhancement of the inflammatory status[221]. Based on that, while MCJ seems to contribute to disease development in the liver, the opposite pattern appears to take place in the gut, suggesting a tissue-dependent activity of the protein. Nevertheless, more research in the field is needed to elucidate the specific mechanistic relationship between MCJ and metabolism, specifically between MCJ and NAFLD, as well as to consider its silencing as a feasible and realistic therapeutic strategy.

4. THERAPEUTIC STRATEGIES IN THE MANAGEMENT OF OBESITY AND NAFLD: GUT MICROBIOTA MODULATION

The important role of gut microbiota in obesity and NAFLD development, explained in the previous chapter, has set out a research field in which possible alternative treatments for these pathologies could be found. The common characteristic of all of these emerging treatments is the ability to modulate the gut microbiota and to restore the dysbiosis through different strategies or mechanisms. In the following section, probiotics, prebiotics, synbiotics and faecal microbiota transplantation strategies are extensively explained due to their narrow relationship with the present PhD Thesis (**Figure 18**).

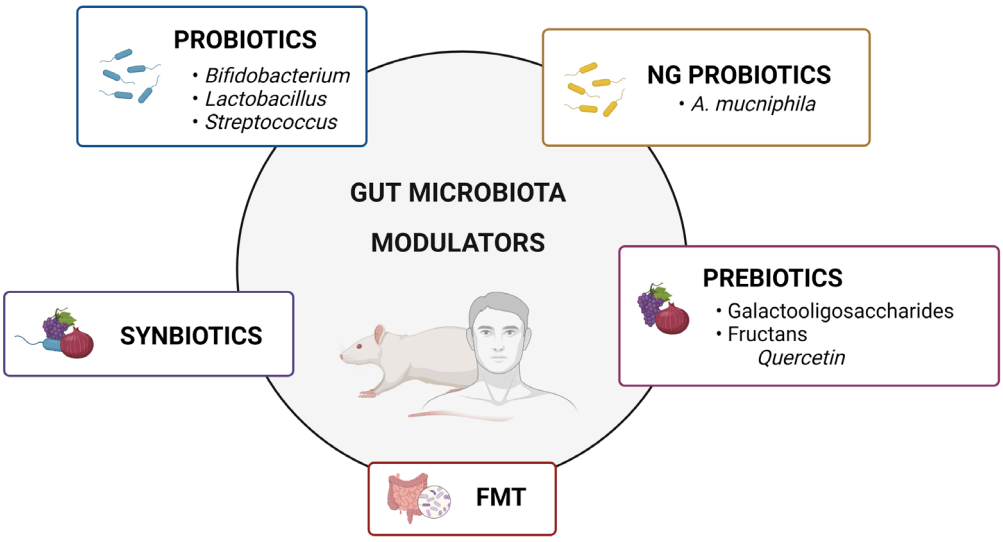


Figure 18. Main therapeutic strategies based on gut microbiota modulation. Probiotics, next-generation probiotics, prebiotics, synbiotics and faecal microbiota transplantation are the main potential therapeutic strategies to face obesity and NAFLD based on gut microbiota modulation. FMT, faecal microbiota transplantation; NG, next-generation.

4.1. Probiotics, Prebiotics and Synbiotics

In the last years, the use of probiotics, prebiotics and synbiotics has emerged as a potential therapeutic alternative for obesity and NAFLD, mainly due to their ability to modulate gut microbiota.

First, probiotics are defined as a group of living microorganisms that are able to colonize the gut and exert beneficial effects to the host[222]. The most common used probiotics are *Bifidobacterium, Lactobacillus* and some strains of *Streptococcus*, such as *Streptococcus thermophilus*[223]. Both animal and clinical studies have shown the potential of probiotics to compete with pathogens, improve gut permeability and modulate the immune system and the inflammatory status[224]. In obesity, while the majority of clinical trials and systematic reviews confirmed that probiotics reduce BMI and adipose mass[225–230], others showed no significant effects. For example, the systematic review carried out by Crovesy *et al.* revealed that, among 14 clinical trials, three did not observe any significant effect, while nine showed an improve in body weight and body fat mass[231]. These differences could be attributed to the sample size or due to methodological issues, being necessary to perform larger studies to elucidate not only the mechanisms under these effects, but also to assure their safety and their effectiveness[222]. Regarding NAFLD, the use of probiotics has been related to a decrease in fat accumulation, oxidative stress, inflammation, and fibrosis[224,232]. As our group previously reported, different animal

experiments have proved the benefits of probiotics in NAFLD development[95]. Concerning clinical trials, the benefits have also been pointed out. For example, the use of VSL#3, a probiotic mix of eight different bacteria, has shown promising results both in animal and human studies, reducing inflammation, improving liver status and insulin sensitivity, and preventing liver fibrosis[233,234]. In fact, supplementation with VSL#3 during four months in children with NAFLD increases GLP-1 levels, suggesting that the improvement in BMI and fatty status could be exerted by this signaling pathway[235]. Moreover, children with NAFLD and obesity showed an improvement in liver enzymes and lipid profile after probiotic supplementation[236]. The systematic review and meta-analysis performed by Xiao *et al*. demonstrated the beneficial effects of probiotics in NAFLD development, suggesting its use as a potential complementary treatment[237]. However, as it occurred with obesity, larger and well-designed studies are needed in order to prove the clinical efficacy of probiotics in NAFLD[238].

In the last years, the next-generation probiotics have emerged, defined as living microorganisms that have been identified in commensal microbiota analysis and that confer a benefit to the host health when are administered in adequate amounts[157]. *Faecalibacterium prausnitzii, Akkermansia muciniphila* or some strains from *Clostridia* genus could act as next-generation probiotics. For example, *F. prausniztii* is a butyrate producer that has anti-inflammatory properties and whose abundance is decreased in patients with obesity[222,239]. Moreover, *A. muciniphila* is an anaerobic bacterium specialized in mucin degradation, whose abundance has been related to a healthy metabolic status[240]. In fact, patients with obesity and NAFLD have been reported to present lower amounts of this microorganism[241]. The daily supplementation of HFD-fed mice with *A. muciniphila* has demonstrated to counteract obesity and reduce fat mass, insulin resistance and lipid metabolism alteration[242]. Additionally, the administration of lived or pasteurized *A. muciniphila* to patients with obesity for three months was safe, well tolerated and was able to improve insulin sensitivity, lipid profile, anthropometric measurements, and inflammatory status[243]. Based on these metabolic beneficial effects, *A. muciniphila* has been proposed as a promising probiotic[244]. Recently, pasteurized *A. muciniphila* has been approved by the EFSA as a food ingredient, after a toxicological and safety analysis[245,246], and different clinical trials are evaluating its potential beneficial effects in metabolic diseases.

Secondly, prebiotics are non-digestible food ingredients that improve host health by favoring the growth of beneficial bacteria[247]. These indigestible compounds, mainly carbohydrates, are metabolized in the colon by the gut microbiota, producing SCFAs, gases and other products that improve the gut environment and, consequently, the host health[248]. These microbial modulators can be found naturally in different ingredients, such as legumes, vegetables, or cereals[222]. The most used prebiotics in NAFLD and obesity are galactooligosaccharides and fructans[180], which promote *Bifidobacterium* and *Lactobacillus* growth[222]. In this sense, it is worth it to mention the role of polyphenols, plant-derived compounds with demonstrated beneficial effects in NAFLD on *in vivo* experiments due to their ability to modulate gut microbiota and gut-liver axis[157]. Flavonoids are the most studied polyphenols in metabolic disorders, as they exert anti-inflammatory, antioxidant and prebiotic effects[249]. In fact, quercetin, a type of flavonoid, has been profoundly studied in our research group due to its significant beneficial effects both *in vivo* and *in vitro* studies[250,251]. Quercetin is naturally found in vegetables or fruits such as onions or grapes and constitutes one of the most consumed flavonoids in human diets. Quercetin has shown anti-inflammatory, immunomodulatory, antioxidant and antifibrotic properties, as well as an important role in lipid metabolism regulation, being considered as a potential supplement to face NAFLD development with promising results[252–254]. In fact, quercetin was able to prevent NAFLD progression through the regulation of inflammation, oxidative stress and lipid metabolism in mice fed with methionine- and choline-deficient diet[250]. Additionally, quercetin also modulates gut microbiota composition, exerting prebiotic effects that could be linked with its metabolic benefits, although its mechanism of action is still unclear[251,255,256].

Turning back to prebiotics, while the majority of the experiments performed in animal models showed significant and beneficial effects in metabolic health, the obtained results in the clinical trials are slight and inconclusive[257]. In fact, in obesity, minor or no beneficial effects in BMI and lipid profile have been observed in patients with obesity under prebiotic administration[222]. Additionally, inulin has been related to a decrease in appetite and food intake in patients with obesity, although no effects on BMI or body weight were reported[159]. Clinical trials and animal experiments in NAFLD development have shown better results: an improvement in liver enzymes levels, inflammation, and lipid accumulation, as well as a reduction in appetite and *de novo* lipogenesis have been observed with prebiotics[223,258]. Furthermore, prebiotics improve gut barrier status, induce

Faecalibacterium prausnitzii growth, and decrease LPS concentration[180,233]. In the clinical trial performed by Bomhof *et al*., an improvement in steatosis and fasting glucose were observed in the group of NASH patients subjected to prebiotic supplementation. However, the small sample (14 patients) limited the extrapolation of the study[259]. Moreover, the meta-analysis carried out by Xing *et al*., in which 1555 patients with NAFLD were included, showed that probiotics have better efficacy than prebiotics or synbiotics, although all these strategies are able to decrease hepatic steatosis and improve the lipid profile, the liver enzymes, and the insulin sensitivity in NAFLD, without significantly modifying body weight or hepatic fibrosis[247]. This result was also proven by the analysis performed by Michels *et al*., in which probiotic effects were higher than prebiotics or synbiotics regarding blood pressure indicators and parameters related to liver function[260]. Altogether, there is still a controversy about the efficacy of prebiotics in NAFLD and obesity, being necessary more high-quality clinical trials in the future to ensure not only the effectiveness, but also the duration, the type of prebiotics or its safety[157].

Lastly, synbiotics could be easily defined as the combination of probiotics and prebiotics, although a more accurate definition has been provided by the International Scientific Association for Probiotics and Prebiotics: 'a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host'[261]. In this sense, synbiotics could be classified into synergistic, in which the prebiotic acts as a substrate for the administered probiotic; or complementary, in which the combination of prebiotic and probiotic aims to affect and improve the host microbial environment[262]. Different complex combinations of bacterial strains and dosages of diverse prebiotics have been tested in animal and human studies and, although *in vitro* experiments have shown a synergistic effect of the synbiotics compared with prebiotics or probiotics alone, this potential outcome is not always reproducible in *in vivo* experiments and clinical trials. Regarding obesity, synbiotics have been related to a decrease in BMI, an improvement in lipid profile and in oxidative stress in children with obesity[263,264], results that were similar in adults[262]. However, the available results are conflictive, since some meta-analyses showed an improvement in BMI and body weight with synbiotic administration in patients with obesity, while others reported no significant changes[159,222]. These discrepancies could be attributed to the differences in the synbiotic used, as well as in the dosages or duration of the treatment. In this sense, other effects

such as an improvement in lipid profiles or psychological status have been related to synbiotic supplementation[159]. Concerning NAFLD, synbiotic therapy has been related to an improvement in glucose metabolism, inflammation, and lipid metabolism[232,233]. In fact, synbiotic supplementation together with lifestyle modification has proved to be more effective than the lifestyle modification alone in NAFLD patients, improving inflammatory and hepatic status and reducing BMI[265,266]. Contrarily, a clinical trial showed that, although synbiotic administration significantly modified gut microbiota composition, it did not have any effect on hepatic steatosis and hepatic fibrosis in patients with NAFLD[267]. Furthermore, the combination of five probiotic species, including *Akkermansia muciniphila*, with inulin improved the glucose metabolism in patients with T2DM[268], highlighting the potential of the next-generation probiotics in combination with specific prebiotics to face metabolic diseases.

In summary, probiotics, prebiotics and synbiotics could constitute a feasible strategy to face obesity and NAFLD, although larger and more accurate studies are needed in order to elucidate their mechanisms, safety, and effectiveness, as well as to determine the best dosage or treatment duration.

4.2. Faecal Microbiota Transplantation (FMT)

Faecal microbiota transplantation (FMT) consists in the transference of faecal microbial community from a healthy donor to a recipient with dysbiosis in order to restore their gut microbiota composition. This technique has constituted the most accurate one to demonstrate the causative role of the gut microbiota in health and disease[226,269], and, in the last years, has been pointed out as a potential therapeutic strategy. In fact, it is currently well implemented for the treatment of *Clostridium difficile* infections, leading to the cure of the disease in 85% of the cases[257]. Therefore, FMT is contemplated in the medical guidelines for the treatment of refractory and recurrent *C. difficile* infections[270]. Regarding the different administration routes, FMT can be performed by two types of methods: lower (colonoscopy, enema) and upper gastrointestinal routes (nanojejunal, nasoduodenal or nasogastric tube and oral capsules)[257,271]. In this sense, Yu *et al.* developed a safety and efficient FMT capsules for *Clostridium difficile* infection that let the therapy to be administered repetitively without a significant invasiveness[272]. The performed studies have notified a good tolerance and safety, as seldom unfavourable events have occurred[257]. However, the American Gastroenterological Association is now

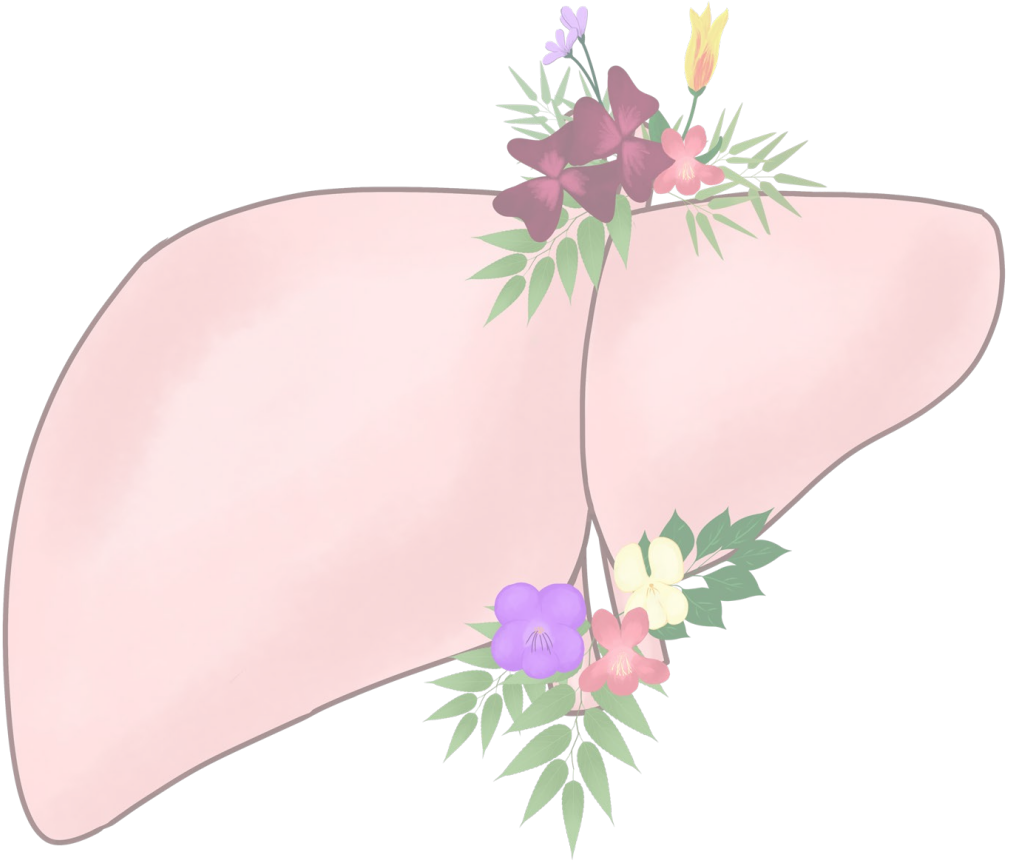
performing a long-term registry to evaluate the safety of the FMT over time[110]. Furthermore, despite the use of FMT as an effective strategy, there is a lack of standardization that should be addressed, as there is no consensus about the screening and selection of the donor, the processing sample, or the number of doses, being necessary to establish universal standardized guidelines to guarantee the safety and the efficacy of the process[110,270].

Based on the relationship of the gut microbiota and many diseases and considering the efficacy of FMT with *C. difficile* infections, different studies are focused on the potential of FMT in other illnesses such as NAFLD or obesity, although it is still not considered in the clinical practice[270].

Concerning obesity, not only the aforementioned experiments performed by Bäckhed *et al.*[139] and Turnbaugh *et al.*[140,141] with FMT in animal models, but also other following studies have demonstrated the potential of this strategy as an alternative therapy in the management of the disease[144,273]. All these findings have set the basis for the development of human studies in order to elucidate the real potential of FMT. The first clinical trials were performed by Vrieze *et al.* and Kootte *et al*., who showed an improvement of insulin sensitivity and glycated haemoglobin in patients with obesity after six weeks of FMT from selected healthy donors compared to the placebo (autologous FMT). Moreover, Vrieze *et al.* found that patients that had received the FMT from healthy donors had a higher abundance of butyrate-producing bacteria, suggesting a role of this SCFA in insulin metabolism. However, these differences disappeared at the 18th week post transplantation, suggesting that the effect of the treatment is mainly at short term. Additionally, both studies only included men, being necessary to analyse this approach in women[274,275]. Moreover, in adolescents, although a reduction in abdominal adiposity was observed, no effect on BMI was detected after FMT[276]. Other authors have not reported any modification of glucose or lipid metabolism after FMT both in woman and man adults[272,277,278]. In this sense, the clinical trial carried out by Mocanu *et al*. showed that the combination of FMT-capsules and the fibre supplementation was able to improve insulin sensitivity as well as increase bacterial richness[279], suggesting that the combinatory strategy could be beneficial for obesity care. Moreover, it is worth it to mention the effect of FMT from donors that have previously been under bariatric surgery to patients with obesity, as showed an improvement in insulin sensitivity[280]. Altogether, based on the systematic review performed by Zhang *et al*., FMT needs to be further

investigated in order to consider it a potential therapy, as beneficial observed effects on glucose metabolism are not maintained at long term, as well as no differences on BMI or cholesterol-related parameters have been observed yet[281]. Considering that the clinical studies that have been carried out until now involve a small sample size, larger studies with mixed gender would be needed to further analyse the effect of FMT on obesity development, as well as to deepen in the mechanistic pathways involved[226,270].

The therapeutic potential of FMT has been also explored in liver diseases and, as in obesity, findings in animal studies have set the foundation of this tool as an alternative treatment[95,156,282]. In humans, FMT was able to reduce hepatic complications related to hepatic encephalopathy in patients[283], reason of which this strategy was considered as a feasible alternative in NAFLD development. Currently, there are eleven clinical trials related to NAFLD and FMT: two of them have been already completed, while the status of five of them is unknown. In general, these clinical trials have highlighted a beneficial effect of the treatment, increasing insulin sensitivity and SCFAs production, as well as shaping gut microbiota composition[284]. In this regard, Craven *et al*. demonstrated an improvement in the gut permeability after FMT procedure, although no differences in insulin sensitivity or hepatic damage were observed[285]. Other studies have also pointed out an improvement in plasma metabolites or steatohepatitis-related parameters[286,287]. Moreover, it has been reported that the efficacy of FMT is higher in lean NAFLD than in obese NAFLD[287], finding that is in consensus with the available results of the clinical trials performed in both diseases. Altogether, the different clinical trials that have analysed the effect of FMT on NAFLD development have shown a good efficacy of the treatment, observing an improvement of glucose metabolism and liver steatosis[224]. Although the mechanistic action of FMT in NAFLD development is still unknown and more and larger studies are needed, the results point out to the evidence of its use as a potential alternative for the disease management[224].



AIMS

Considering the demonstrated important contribution of the intestinal microbiota in obesity and non-alcoholic fatty liver disease (NAFLD), as well as the limited treatments to face these pathologies, **the main aim** of this PhD Thesis is to elucidate the mechanistic role of gut microbiota in the pathogenesis of obesity and NAFLD, and to investigate the modulation of the gut microbiota as an alternative strategy in the treatment of these diseases.

The Thesis is organized in three studies with the above-mentioned and common purpose. The nucleus of the first study is bariatric surgery, the gold-standard technique to address severe obesity, analysing the long-term effects that this treatment has on gut microbiota, and the link between this microbiota modulation and the improvement of the disease in patients. Accordingly, the second study focuses on the modulation of the gut microbiota by the combinatory use of a nutritional intervention, the first line of action in these diseases, and the synbiotic constituted by quercetin and the bacterium *Akkermansia muciniphila,* as a therapeutic strategy in the management of obesity and NAFLD in a rat model. In line with the former two investigations, the third study aims to evaluate the relationship between the intestinal microbiota and the mitochondrial function in the development of non-alcoholic steatohepatitis (NASH) in a mouse model, as well as to determine the therapeutic potential of the microbiota-mitochondria crosstalk in the disease.

Thus, to reach the main purpose of this PhD Thesis, the following **specific objectives** are considered:

» **First research objective**

To study the long-term effects of bariatric surgery, the gold-standard treatment of obesity, on the composition and functionality of gut microbiota in patients with clinical severe obesity.

» **Second research objective**

To investigate the link between the gut microbiota profile shaped by the bariatric surgery at long term and the recovery of the patients with clinical severe obesity.

» **Third research objective**

To analyse the combination of a nutritional intervention, one of the first approaches in the treatment of obesity and NAFLD, and the potential synbiotic constituted by quercetin and *Akkermansia muciniphila* in the progression of the diseases in a rat model.

» **Fourth research objective**

To determine the relationship between the modulation of gut microbiota by the potential synbiotic combination of quercetin and *Akkermansia muciniphila* and the beneficial observed effects in a rat model of early obesity and NAFLD.

» **Fifth research objective**

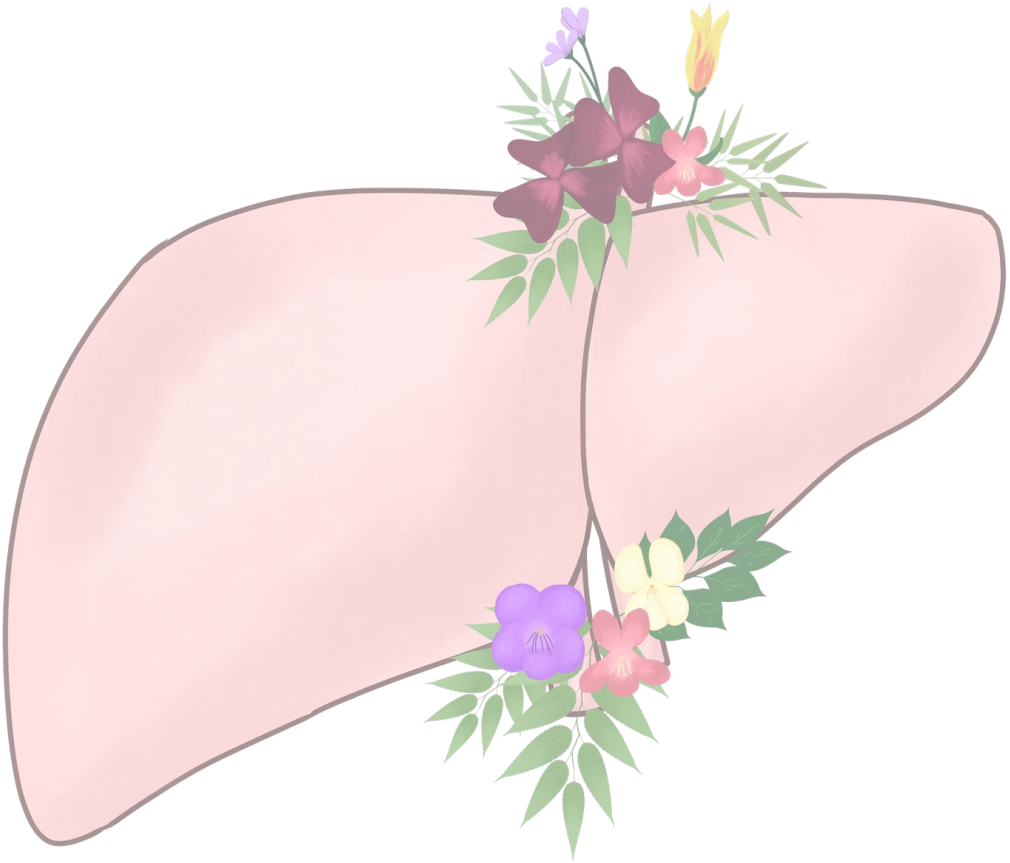
To evaluate the effect of an enhanced mitochondrial activity by the deficiency of methylation-controlled J (MCJ) protein in the development of the disease in a mouse model of NASH.

» **Sixth research objective**

To study the possible link between MCJ deficiency and gut microbiota composition and functionality in a mouse model of NASH.

» **Seventh research objective**

To determine if the observed effects associated with the MCJ deficiency in the mouse model of NASH can be transferred to germ-free mice through caecal microbiota transplantation.



PUBLICATIONS

The current PhD Thesis entitled "*Gut microbiota modulation as an alternative in the management of non-alcoholic fatty liver disease and obesity*" is presented by compendium of publications, and includes a total of three published articles which information is detailed below:

» **First publication.** 'Long-term effects of bariatric surgery on gut microbiota composition and faecal metabolome related to obesity remission'.

Available from: https://www.mdpi.com/2072-6643/13/8/2519

» **Second publication.** 'The synbiotic combination of *Akkermansia muciniphila* and quercetin ameliorates early obesity and NAFLD through gut microbiota reshaping and bile acid metabolism modulation'.

Available from: https://www.mdpi.com/2076-3921/10/12/2001

» **Third publication.** 'Enhanced mitochondrial activity reshapes a gut microbiota profile that delays NASH progression'.

Available from: https://aasldpubs.onlinelibrary.wiley.com/doi/10.1002/hep.32705

A copy of each one of the three articles, together with their supplementary material, as well as a schematic prelude that specifically link the contributions are included below.

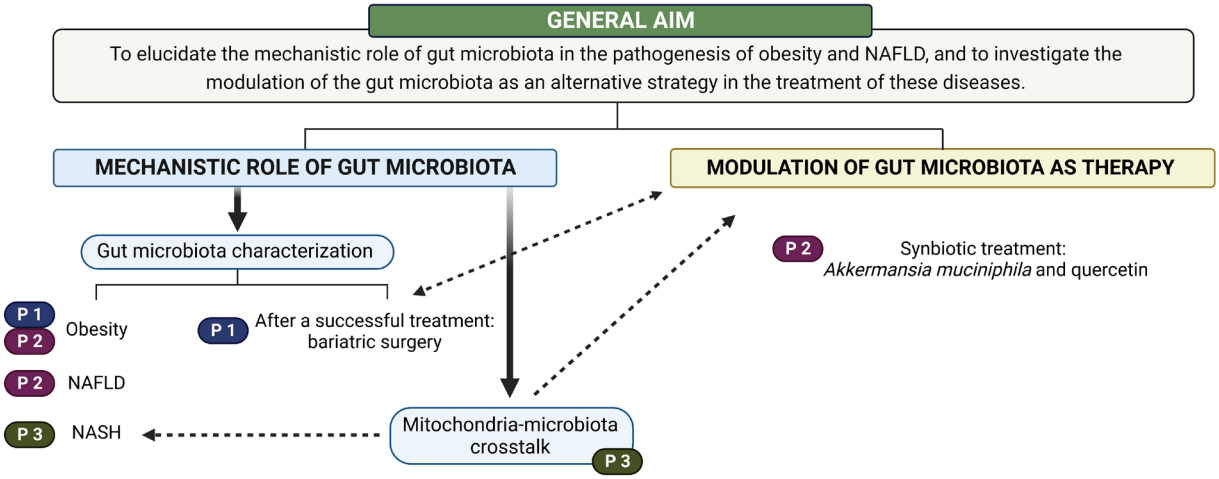


Figure 19. Schematic representation of the general aim of the PhD Thesis. The aim of the present PhD Thesis could be divided into two different sections of knowledge in which the three research articles are included: one on side, studying the mechanistic role of the gut microbiota in obesity and NAFLD and, on the other side, elucidate the potential of the modulation of the gut microbiota as a feasible strategy in the management of these diseases. In order to determine the mechanistic role of the gut microbiota in the

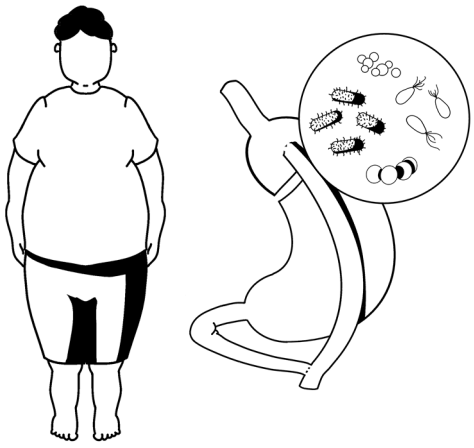
disease, a characterization of the gut microbiota composition and functionality, not only in a disease status, but also after the success of a treatment, needs to be addressed. Here, the first publication tries to characterize the gut microbiota profile in severe obesity, as well as after a successful bariatric surgery. Thus, the analysis of the difference between these two characterizations could point out new insights in the therapeutic field. The second publication focuses on gut microbiota characterization in early obesity and NAFLD, as well as on the combination of quercetin and *Akkermnasia muciniphila* as a feasible synbiotic for the treatment of these diseases. The third publication delves into one of the mechanistic roles of the gut microbiota: the mitochondria-microbiota crosstalk, in addition to characterizing the gut microbiota profile related to NASH. NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; P1, first publication; P2, second publication; P3, third publication.

FIRST PUBLICATION

‘Long-term effects of bariatric surgery on gut microbiota composition and faecal metabolome related to obesity remission’

Juárez-Fernández M, Román-Sagüillo S, Porras D, García-Mediavilla MV, Linares P, Ballesteros-Pomar MD, Urioste-Fondo A, Álvarez-Cuenllas B, González-Gallego J, Sánchez-Campos S, Jorquera F, Nistal E.

Nutrients (2021) 13(8):2519. DOI: https://doi.org/10.3390/nu13082519



SECOND PUBLICATION

'The synbiotic combination of *Akkermansia muciniphila* and quercetin ameliorates early obesity and NAFLD through gut microbiota reshaping and bile acid metabolism modulation'

Juárez-Fernández M, Porras D, Petrov P, García-Mediavilla MV, Román-Sagüillo S, Soluyanova P, Martínez-Flórez S, González-Gallego J, Nistal E, Jover R, Sánchez-Campos S.

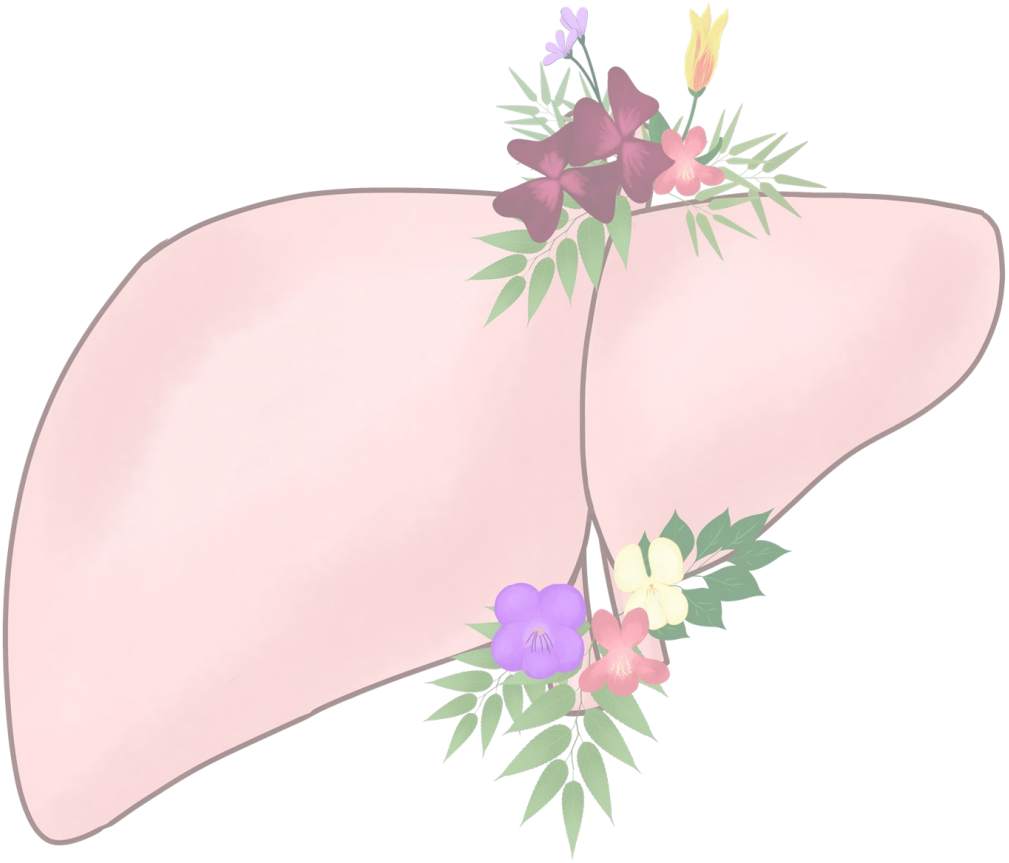
Antioxidants (2021) 10(12):2001. DOI: https://doi.org/10.3390/antiox10122001

THIRD PUBLICATION

‘Enhanced mitochondrial activity reshapes a gut microbiota profile that delays NASH progression’

Juárez-Fernández M*, Goikoetxea-Usandizaga N*, Porras D, García-Mediavilla MV, Bravo M, Serrano-Maciá M, Simón J, C. Delgado T, Lachiondo-Ortega S, Martínez-Flórez S, Lorenzo O, Rincón M, Varela-Rey M, Abecia L, Rodríguez H, Anguita J, Nistal E, Martínez-Chantar ML, Sánchez-Campos, S.

Hepatology (2022) 00:1-16. DOI: https://doi.org/10.1002/hep.32705



INTEGRATIVE VIEW OF THE PhD THESIS

Nowadays, obesity and NAFLD are two of the most important public health concerns worldwide. The lack of an effective treatment to face them, along with the current sedentary lifestyle and the intake of high-fat and high-sugar diets have led to an increasing trend in the prevalence of both diseases, with shocking future estimations. In the last years, gut microbiota has been identified as a key contributor factor to the development of these diseases, so it has become a research field not only in the characterization of the pathogenic mechanisms, but also in the hunt for new therapeutic alternatives. In this sense, the modulation of gut microbiota through different strategies such as fecal microbiota transplantation or the administration of probiotics, prebiotics, and synbiotics are being considered as possible treatments to face both obesity and NAFLD. In the present PhD Thesis, the general aim is to identify the role of the gut microbiota in the pathogenesis of these two diseases, as well as to explore the therapeutic potential of gut microbiota modulation. In this brief chapter, an integrative point of view of the three research articles that constitute this PhD Thesis is detailed.

Firstly, one of the essential tools aimed to elucidate the role of gut microbiota in obesity and NAFLD development is based on the characterization of the disease-related microbial profiles, in order not only to identify a common pattern, but also to establish relationships between the abundance of certain bacterial taxa, their metabolic functions, and the consequent activation or inactivation of different signaling pathways related to the disease progression. Thus, several results obtained in this PhD Thesis have focused on this feature, characterizing the microbial profile in severe obesity (first publication), early obesity and NAFLD (second publication), and steatohepatitis or NASH (third publication). Even though this characterization has been carried out in patients and in different experimental models (rats and mice), respectively, the methodology used has been consistent, and the microbial profiles that have been identified agree with previous findings, as can be perceived in the corresponding discussion of each article. Furthermore, this characterization has been related to the gut microbiota functionality by fecal metabolomic studies to delve into the physiopathological role that a particular microbial profile associated with the disease could be playing.

Another key point to explore about the pathogenic mechanisms of obesity and NAFLD associated with the gut microbiota is the establishment of relationships between the resulting microbial profile of a successful therapeutic treatment and the improvement of the disease. Additionally, this resulting profile could be also compared with the

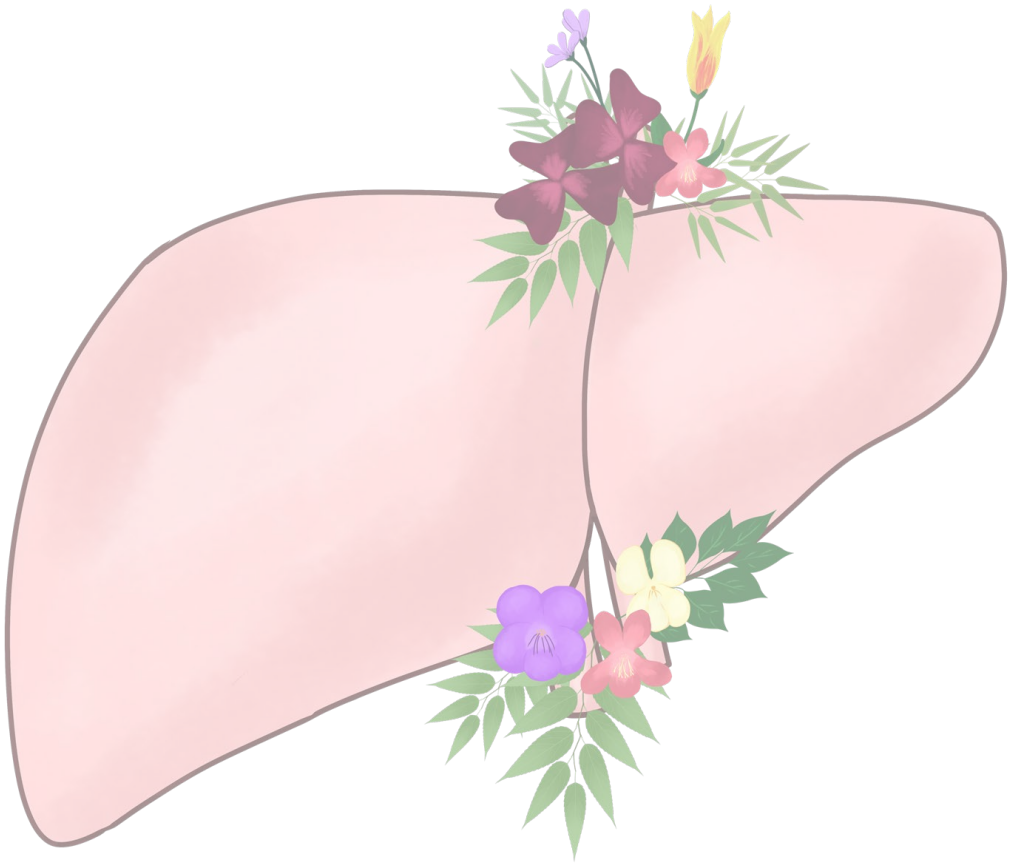
microbial pattern associated with the disease. In this sense, although different treatments may have a specific modulatory effect on gut microbiota, this characterization, in general terms, let us better understand the link between the gut microbiota and the pathogenic mechanisms, as well as to lay the foundations for future treatments. Thus, in the first scientific article of this PhD Thesis, we focused not only on characterizing the gut microbiota in patients with severe obesity, but also on identifying the resulting compositional and metabolomic profile four years after bariatric surgery, one of the most effective therapeutic strategies for the treatment of this disease. This analysis has allowed us to determine the long-term modulatory effect of bariatric surgery, leading to a compositional and metabolomic microbial profile specialized in promoting a lower energy extraction from food, which is one of the metabolic effects involved in body weight loss and, consequently, in the success of the treatment. These results point out the importance of designing new therapeutic alternatives able to act at different points, being one of them, undoubtedly, the gut microbiota.

In addition to the metagenomic and metabolomic characterization, and given the wide role that gut microbiota plays in physiological homeostasis, it is also necessary to establish the relationships that this "alive organ" has with the whole organism. Thus, in recent years, a bidirectional link between gut microbiota and mitochondria has been suggested, and different studies have proposed that this relationship could also be involved in liver diseases. For this reason, in our third study, we wanted to delve into this microbiota-mitochondria crosstalk. Therefore, using a genetic murine model with mitochondrial hyperactivity in combination with a specific diet, we developed a mice model of steatohepatitis or NASH. We demonstrated that the mitochondrial hyperactivity has a protective effect on the liver, avoiding NASH development. However, we wondered whether the gut microbiota participated in this protective effect and, if so*, what role did the gut microbiota play?* Based on these scientific questions, we carried out the 'gold-standard' methodology for the demonstration of the causal role of the gut microbiota: fecal microbiota transplantation. Thus, we observed that the gut microbiota profile associated with the mitochondrial hyperactivity is capable of transferring this protective effect to germ-free mice, participating directly in an anti-inflammatory and antifibrotic response that halts steatohepatitis progression. These results point out to the great potential of the gut microbiota in health and disease, demonstrating the close relationship between the microbiota and mitochondria, and opening up to a new possible research field

for therapeutic approaches based on the development of specific microbial profiles with specific metabolic characteristics.

Lastly, and in line with the general aim of this PhD Thesis, we wanted to evaluate the gut microbiota modulation as a therapeutic alternative in the treatment of obesity and NAFLD. Considering the results of the first publication, in which we proved the modulatory effect on gut microbiota composition of a successful treatment such as bariatric surgery, we wanted to verify whether such modulation could have beneficial effects related to disease remission. Thus, the first general strategy in the treatment of both obesity and NAFLD is a lifestyle change towards nutritional interventions, which commonly fails due to the low adherence in patients. Therefore, in our second scientific article, we wanted to answer the following question: *would the modulation of the gut microbiota through the administration of a probiotic and/or prebiotic compound improve or enhance the beneficial effects of the nutritional intervention?* Thus, we combined in a feasible synbiotic a natural and prebiotic compound, such as quercetin, extensively studied by our research group, and a bacterium with demonstrated metabolic benefits such as *Akkermansia muciniphila*. This potential symbiotic, in combination with the nutritional strategy, improved the metabolic and inflammatory state associated with obesity and NAFLD in a diet-induced animal model. These results open up to a range of therapeutic possibilities to face these diseases, highlighting the importance of combined treatments that are able to exert additional beneficial effects, both in the short and long term, and consequently increase the possibilities of disease remission and cure.

In summary, this PhD Thesis is constituted by three scientific articles focused on the gut microbiota and its relationship with obesity and NAFLD. This project not only characterized the microbial profile associated with the diseases, but also considered the modulation of this altered profile as a possible therapeutic alternative that could be used in the future, either alone or in combination with other strategies to address these two current public health concerns. However, more studies are needed to delve into the detailed role of the gut microbiota in the pathogenesis of the disease, as well as further research and clinical trials to contribute to elucidate the effectiveness and safety of these therapeutic alternatives based on gut microbiota modulation.



CONCLUSIONS

Based on the obtained results, as well as on the established aims, the conclusions that have been stated in this PhD Thesis are listed below:

» **First conclusion**

Bariatric surgery has a long-term modulatory effect on gut microbiota composition and functionality in patients with clinical severe obesity.

» **Second conclusion**

The gut microbiota profile shaped by bariatric surgery after four years of the intervention is linked with an improvement in the disease-related status of the patients, being a less efficiency in the energy extraction procedure from the diet the possible mechanism associated with the persistent positive observed effects.

» **Third conclusion**

The combination of a nutritional intervention together with the administration of the potential synbiotic constituted by quercetin and *Akkermansia muciniphila* have beneficial effects that contribute to delay the disease progression in a rat model of early obesity and NAFLD.

» **Fourth conclusion**

The modulation of the gut microbiota due to the combination of nutritional intervention and administration of quercetin and *Akkermansia muciniphila* results in bile acid metabolism regulation, as well as the modulation of the inflammatory status in the liver and the lipid metabolism in adipose tissue. These main targets could be involved in the ability of the potential synbiotic combined with the nutritional intervention to counteract early obesity and NAFLD development.

» **Fifth conclusion**

The deficiency of methylation-controlled J (MCJ) protein confers an improved mitochondrial activity that is able to delay the progression of the disease in a NASH-lean mouse model.

» **Sixth conclusion**

The MCJ deficiency modulates gut microbiota composition and functionality, conferring a specific gut microbiome profile together with an improved gut-liver axis and gut barrier permeability that could be related to the beneficial effects observed in NASH development.

» **Seventh conclusion**

The specific gut microbiota profile shaped by MCJ deficiency is able to be transferred to germ-free mice through caecal microbiota transplantation and to exerts the protective effects that contribute to delay NASH progression.

> GENERAL CONCLUSION <

Gut microbiota plays an essential role in the pathogenesis and development of obesity and non-alcoholic fatty liver disease. Thus, the modulation of gut microbiota not only as a consequence of current treatments like bariatric surgery, but also through the mitochondria-microbiota crosstalk or other strategies such as synbiotic administration has significant beneficial effects in the development of these diseases and could be considered as an alternative therapeutic approach. Nevertheless, the specific mechanisms and signaling pathways in which gut microbiota modulation exerts its benefits remain unclear, so delve into these processes is essential to propose safe, consistent, and effective treatments that could be designed and combined based on the characteristics of each patient.

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TESIS DOCTORAL

MODULACIÓN DE LA MICROBIOTA INTESTINAL COMO ALTERNATIVA TERAPÉUTICA EN EL MANEJO DE LA ENFERMEDAD DE HÍGADO GRASO NO ALCOHÓLICO Y LA OBESIDAD

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León, 2023

ABSTRACT IN SPANISH – RESUMEN EN ESPAÑOL

Estado del arte

La obesidad constituye hoy en día uno de los principales problemas de salud pública en el mundo, siendo considerada una pandemia que afecta no sólo a la población adulta sino también a la población infantil, con una prevalencia mundial de aproximadamente el 25%[3,5]. La obesidad, definida como la acumulación excesiva de grasa en el cuerpo, es una enfermedad multifactorial originada como consecuencia de un balance positivo a largo plazo de la energía ingerida en detrimento del gasto energético[3,17]. Sin embargo, varios factores como factores genéticos, epigenéticos, nutricionales, hormonales, metabólicos, psicológicos o incluso el estatus socioeconómico y el estilo de vida de cada individuo influyen en la aparición y desarrollo de esta enfermedad[18,19]. Un cambio en el estilo de vida a través de planes nutricionales y la realización de ejercicio son el principal abordaje a llevar a cabo en el tratamiento de esta enfermedad[27,28]. Sin embargo, esta estrategia puede no ser efectiva o suficiente, razón por la cual el tratamiento farmacológico constituye una alternativa esencial[13]. A su vez, el tratamiento quirúrgico, comúnmente conocido como cirugía bariátrica, es la herramienta terapéutica por excelencia para casos de obesidad clínica severa[30]. Por último, esta enfermedad se asocia no sólo con una elevada mortalidad y una carga económica para los sistemas públicos de salud mundiales, sino también con una alta morbilidad[6,9]. Así, diferentes enfermedades como enfermedades cardiovasculares, diabetes, cáncer o enfermedad de hígado graso no alcohólico (NAFLD, por sus siglas en inglés) se han asociado con la obesidad[4,14].

NAFLD es una enfermedad caracterizada por la acumulación de grasa en el hígado por causas no relacionadas con el consumo de alcohol, con infecciones virales, o con la ingesta de fármacos, y constituye a día de hoy la enfermedad hepática crónica más prevalente[35,37]. Esta se inicia con la esteatosis simple, una condición benigna caracterizada por la acumulación de grasa en más de un 5% de los hepatocitos, la cual puede progresar hacia esteatohepatitis (NASH, por sus siglas en inglés), condición patológica que se caracteriza por la presencia de inflamación y daño hepático, e incluso hacia fibrosis, cirrosis o hepatocarcinoma[37,38]. Con una prevalencia del 25% a nivel mundial[41], las estimaciones acerca de la tendencia de esta enfermedad son alarmantes, tanto en adultos como en adolescentes y niños[45]. De hecho, NAFLD es considerada la manifestación hepática del síndrome metabólico y posee una estrecha relación con la

obesidad, mostrando ambas enfermedades tendencias crecientes y similares[47,48]. NAFLD es una enfermedad multifactorial cuya patogénesis, según la teoría del múltiple impacto, es un proceso complejo en el que diferentes factores, como la alteración del metabolismo lipídico, la resistencia a la insulina, la inflamación, el estrés de retículo endoplásmico, el estrés oxidativo, la disfunción mitocondrial, la alteración de la microbiota intestinal y la disrupción del eje intestino-hígado, junto con la predisposición genética, participan de forma paralela y sinérgica en la aparición y desarrollo de la enfermedad[58,61]. Actualmente, la principal recomendación médica para hacer frente a NAFLD se basa en el cambio en el estilo de vida, implementando estrategias nutricionales y la práctica de ejercicio físico[91]. De este modo, no existe tratamiento farmacológico específico aprobado hoy en día para esta enfermedad, aunque sí existen numerosos fármacos que están siendo investigados para tal fin, principalmente aquellos enfocados al tratamiento de obesidad y diabetes, dada la estrecha relación de NAFLD con estas enfermedades[41,48,97].

En los últimos años, se ha identificado la alteración de la microbiota intestinal como un factor contribuyente a la aparición y desarrollo de obesidad y NAFLD, planteándose incluso posibles alternativas terapéuticas basadas en la modulación de la misma. La microbiota intestinal se define como el conjunto de microorganismos (arqueas, virus, hongos y, mayoritariamente, bacterias) que habitan en el tracto digestivo y que desempeñan un papel esencial para la salud del huésped[108]. De hecho, las funciones de la microbiota intestinal abarcan tanto funciones estructurales, metabólicas, inmunológicas e incluso neurológicas, siendo considerada como un órgano interno indispensable para mantener la homeostasis fisiológica del organismo[111,119,123]. La composición de la microbiota intestinal varía no sólo a lo largo de la vida de un individuo, en la que varios factores como la ingesta de antibióticos, la alimentación o el estrés pueden modificar dicha composición, sino también a lo largo del tracto digestivo, debido principalmente a las diferentes características morfológicas y fisiológicas de cada una de las regiones[105]. Así, pese a que existen patrones generales comunes a la población en relación con la composición de la microbiota intestinal, tanto en la salud como en la enfermedad, cada individuo posee un perfil microbiano único[116,119].

La relación entre las enfermedades metabólicas, principalmente NAFLD y obesidad, y la microbiota intestinal ha sido ampliamente estudiada tanto en modelos animales como en pacientes, demostrando así el papel fundamental que esta es capaz de desempeñar en la predisposición, aparición y desarrollo de estas enfermedades. El papel

de la microbiota intestinal en estas afecciones deriva de la comunicación anatómica existente entre el intestino y el hígado a través de la vena porta, llamada eje intestino-hígado[56]. En un estado de salud, el intestino está provisto de una barrera intestinal que mantiene la homeostasis fisiológica y evita que microorganismos patógenos o metabolitos tóxicos bacterianos puedan alcanzar el hígado y la circulación sistémica[160]. Sin embargo, en condiciones de sedentarismo, dietas ricas en grasas o en estados de enfermedad, se produce una alteración de la composición de la microbiota intestinal que conlleva una alteración de esta barrera intestinal, permitiendo que diferentes productos microbianos puedan alcanzar tanto el hígado como la circulación sistémica, provocando inflamación y daño hepático, y favoreciendo así el desarrollo de la enfermedad[160,164,166]. A su vez, la alteración de la microbiota intestinal se traduce en una alteración de su funcionalidad y, por tanto, en una alteración en la producción de sus metabolitos, los cuales ejercen un papel clave en el desarrollo de estas enfermedades[160]. Recientemente, se ha identificado una estrecha relación entre la microbiota intestinal, los metabolitos microbianos y la mitocondria, orgánulo energético por excelencia. De hecho, esta relación parece estar implicada en el desarrollo de NAFLD y obesidad, aunque son necesarios más estudios para profundizar en los mecanismos específicos que lo ocasionan[212]. En este sentido, la proteína J controlada por metilación (MCJ, por sus siglas en inglés) se ha identificado como un posible campo de investigación. Así, la deficiencia en este regulador negativo de la cadena mitocondrial de transporte de electrones posee efectos beneficiosos en el manejo de diferentes enfermedades hepáticas como NAFLD, e incluso altos niveles de esta proteína han sido identificados en pacientes con NASH, pudiendo ser considerada como una posible vía terapéutica[214,217,220].

Finalmente, dado el papel esencial que ejerce la microbiota intestinal en el desarrollo de obesidad y NAFLD, en los últimos años se han propuesto diferentes estrategias basadas en la modulación de la microbiota intestinal como posibles alternativas terapéuticas para hacer frente a estas enfermedades. La administración de probióticos, prebióticos, simbióticos o el trasplante de microbiota fecal constituyen los principales focos de estudio, con resultados prometedores tanto en modelos animales como en ensayos clínicos[180,247,272]. Sin embargo, se necesita profundizar en los mecanismos de acción que subyacen tras estas estrategias de modulación de la microbiota intestinal, así como esclarecer su efectividad, seguridad, e incluso la dosis o la duración de estos posibles tratamientos.

Objetivos

El principal objetivo de la presente Tesis Doctoral es elucidar el mecanismo mediante el cual la microbiota intestinal participa en la patogénesis de la obesidad y NAFLD, e investigar la modulación de dicha microbiota como una posible alternativa terapéutica para hacer frente a estas enfermedades.

Así pues, para lograr el objetivo general previamente expuesto, se proponen los siguientes objetivos específicos:

» **Primer objetivo**

Estudiar los efectos de la cirugía bariátrica a largo plazo en la composición y funcionalidad de la microbiota intestinal en pacientes con obesidad severa.

» **Segundo objetivo**

Investigar la relación existente entre el perfil microbiano modulado por la cirugía bariátrica a largo plazo y la recuperación de los pacientes con obesidad severa.

» **Tercer objetivo**

Estudiar el efecto combinado de una intervención nutricional, uno de los primeros abordajes terapéuticos para el tratamiento de obesidad y NAFLD, y el posible simbiótico constituido por quercetina y la bacteria *Akkermansia muciniphila* en la progresión de estas enfermedades en un modelo animal.

» **Cuarto objetivo**

Determinar la correlación existente entre la modulación de la microbiota intestinal realizada por el posible simbiótico formado por quercetina y *Akkermansia muciniphila* y los efectos beneficiosos observados en el modelo animal de obesidad temprana y NAFLD.

» **Quinto objetivo**

Evaluar el efecto de una actividad mitocondrial potenciada por la deficiencia de la proteína MCJ en el desarrollo de la enfermedad en un modelo murino de NASH.

» **Sexto objetivo**

Estudiar la posible relación existente entre la deficiencia de la proteína MCJ y la composición y funcionalidad de la microbiota intestinal en un modelo murino de NASH.

» **Séptimo objetivo**

Analizar si los efectos observados asociados a la deficiencia de la proteína MCJ en el modelo murino de NASH pueden ser transferidos a ratones libres de gérmenes a través de un trasplante de microbiota cecal.

Publicaciones

La presente Tesis Doctoral titulada 'Modulación de la microbiota intestinal como alternativa en el manejo de enfermedad de hígado graso no alcohólica y obesidad' se presenta en modalidad por compendio de publicaciones, e incluye un total de tres artículos científicos publicados, cuya información se incluye a continuación.

» **Primera publicación**. **'**Long-term effects of bariatric surgery on gut microbiota composition and faecal metabolome related to obesity remission'.

Disponible en: https://www.mdpi.com/2072-6643/13/8/2519

» **Segunda publicación**. **'**The synbiotic combination of *Akkermansia muciniphila* and quercetin ameliorates early obesity and NAFLD through gut microbiota reshaping and bile acid metabolism modulation'.

Disponible en: https://www.mdpi.com/2076-3921/10/12/2001

» **Tercera publicación**. **'**Enhanced mitochondrial activity reshapes a gut microbiota profile that delays NASH progression'.

Disponible en: https://aasldpubs.onlinelibrary.wiley.com/doi/10.1002/hep.32705

A continuación, se incluye un resumen de cada uno de los tres artículos.

Primera publicación

La obesidad constituye uno de los principales problemas de salud a nivel mundial, siendo la cirugía bariátrica el tratamiento por excelencia cuando las alternativas no quirúrgicas no han surtido efecto. Teniendo en cuenta la estrecha relación entre la alteración de la microbiota intestinal y el desarrollo de obesidad, en los últimos años se ha propuesto la capacidad de la cirugía bariátrica para modular dicha microbiota como uno de los mecanismos subyacentes que contribuyen al éxito terapéutico. En base a ello, el principal objetivo de esta investigación fue evaluar los efectos a largo plazo que posee la cirugía bariátrica sobre la composición y funcionalidad de la microbiota intestinal en pacientes con obesidad severa.

Para alcanzar dicho objetivo, se incluyeron en el estudio un total de nueve pacientes con obesidad severa. Se llevó a cabo una caracterización demográfica y antropométrica y se recogieron muestras de heces y de sangre tanto antes como cuatro años después de la cirugía bariátrica. Así, se determinaron los principales parámetros bioquímicos relacionados con la obesidad, y la composición de la microbiota presente en las heces se analizó mediante secuenciación masiva del ARN 16S, utilizando para ello la plataforma de Illumina MiSeq. Por último, los metabolitos polares se identificaron y cuantificaron en heces mediante cromatografía líquida-espectrometría de masas, mientras que los ácidos grasos de cadena corta (SCFAs, por sus siglas en inglés) se analizaron mediante cromatografía de gases-espectrometría de masas.

Los resultados indicaron, tal y como era de esperar, una mejora significativa de los parámetros bioquímicos y antropométricos en los pacientes cuatro años después de la intervención. La cirugía bariátrica también modificó profundamente la composición de la microbiota intestinal, observándose un aumento de los filos Proteobacteria y Lenthispaerae, así como una reducción del filo Firmicutes. En este sentido, el filo Proteobacteria se ha asociado con una disminución de la inflamación sistémica, una mejora del metabolismo de la glucosa[288], y se ha correlacionado con la pérdida de peso[289]. Además, a nivel de familia, se observó un aumento de la abundancia de Enterobacteriaceae, que se ha correlacionado negativamente con la concentración plasmática de colesterol en humanos[290,291] y positivamente con la pérdida de peso en modelos animales[292].

La cirugía bariátrica también modificó la composición de la microbiota intestinal a nivel de género. Los géneros *Butyricimonas*, *Parabacteroides* y *Slackia* se vieron incrementados, mientras que *Acinetobacter, Coprococcus*, *Lachnospira*, *Lactococcus* y *Phascolarctobacterium* mostraron un patrón opuesto. En este sentido, los altos niveles de *Lactococcus* se han asociado con la obesidad y la insulina plasmática en ayunas[293], mientras que una reducción en *Lachnospira* se ha correlacionado positivamente con el índice de masa corporal, la grasa corporal y la concentración de insulina. Además, pacientes sometidos a una cirugía bariátrica fallida mostraron un sobrecrecimiento de *Acinetobacter*[294], y en nuestro estudio, este género se correlacionó positivamente con la concentración plasmática de lipoproteínas de baja densidad (LDL, por sus siglas en inglés), por lo que la reducción significativa de este podría indicar un mayor éxito en el tratamiento. A su vez, *Parabacteroides* se ha correlacionado negativamente con la concentración de insulina sérica después de la cirugía bariátrica[295] y con el índice de masa corporal en nuestro estudio.

El metaboloma fecal de los pacientes con obesidad severa también se vio modificado significativamente cuatro años después de la cirugía bariátrica. El metil-acetoacetato, el carbamoíl-aspartato y el serinfosfato aumentaron, mientras que los metabolitos como el ácido 5-aminolevulínico, la colina, el ácido cítrico, el ácido málico, la taurina, el N-óxido de trimetilamina (TMAO, por sus siglas en inglés) y el ácido trópico disminuyeron en heces. Además, la intervención quirúrgica redujo el ciclo de los ácidos tricarboxílicos, el metabolismo de la glicina, la serina y la treonina, el metabolismo del glioxilato y el dicarboxilato y el metabolismo de la tirosina. Estos resultados sugieren que los aminoácidos de cadena ramificada y los aminoácidos aromáticos, así como el metabolismo energético, se redujeron con la cirugía bariátrica, hallazgos que se sustentan en investigaciones previas[296–299].

El perfil fecal de SCFAs de los pacientes también se modificó significativamente tras la cirugía bariátrica, observándose una disminución de las concentraciones fecales de acetato, butirato y propionato, resultado que concuerda con estudios previos[300,301]. Estos SCFAs se correlacionaron positivamente con el IMC de los pacientes, lo que refuerza su papel en la obesidad. Además, el acetato, el butirato y el propionato correlacionaron negativamente con los géneros *Butyricimonas* y *Parabacteroides*, y positivamente con el género *Lachnospira*. En conjunto, el análisis de correlación mostró un perfil metagenómico y metabolómico relacionado con la cirugía bariátrica que podría estar

involucrado en los efectos beneficiosos observados sobre los parámetros bioquímicos y antropométricos.

En conclusión, nuestros hallazgos apuntan a la cirugía bariátrica como un modulador a largo plazo de la microbiota intestinal, no solo en su composición sino también en su funcionalidad, promoviendo una extracción de energía menos eficiente de la dieta como un posible mecanismo vinculado a los persistentes efectos beneficiosos a nivel metabólico.

Segunda publicación

La obesidad y NAFLD constituyen a día de hoy un problema de salud pública mundial, siendo la principal estrategia terapéutica de ambas enfermedades un cambio en el estilo de vida hacia un hábito saludable. Sin embargo, en numerosas ocasiones, pacientes con obesidad y NAFLD poseen una baja adherencia a este tipo de intervenciones, siendo necesaria la búsqueda de nuevas alternativas terapéuticas. Dada la estrecha relación entre la alteración de la microbiota intestinal y el desarrollo de estas enfermedades, la administración de probióticos, prebióticos o simbióticos puede constituir una terapia razonable para el tratamiento de estas enfermedades. El flavonoide quercetina, con propiedades antioxidantes y antiinflamatorias, ha demostrado ser capaz de atenuar el desarrollo de NAFLD en modelos animales a través de un posible efecto modulador de la microbiota intestinal[251,255]. A su vez, la bacteria *Akkermansia muciniphila* se ha propuesto recientemente como un posible probiótico debido a su efecto protector en el desarrollo de la obesidad[244]. Así, el objetivo de esta segunda investigación se centra en evaluar los efectos de la combinación de una intervención nutricional junto con la suplementación de quercetina y *A. muciniphila* como posible simbiótico en el desarrollo de obesidad temprana y NAFLD en un modelo *in vivo.*

Para lograr tal objetivo, ratas Wistar macho de 21 días de edad se alimentaron con una dieta control o una dieta rica en grasa (HFD, por sus siglas en inglés) durante 6 semanas. Transcurrido este tiempo, se obtuvieron muestras de heces y sangre y todos los animales se alimentaron con dieta control suplementada o no con quercetina y/o *A. muciniphila* durante tres semanas. Por último, los animales fueron sacrificados y se recogieron apropiadamente las muestras para su posterior análisis.

Tras 6 semanas de alimentación con HFD, los animales mostraron un incremento significativo del peso corporal y de la ingesta calórica en comparación con el grupo control. Además, el análisis bioquímico plasmático mostró alteraciones importantes de la actividad de la enzima hepática alanina aminotransferasa, así como de los valores de colesterol, insulina y HOMA-IR, indicando un posible estado de resistencia a insulina asociado al desarrollo de NAFLD. A su vez, el grupo HFD mostró una menor concentración bacteriana en heces, así como un perfil diferente de microbiota intestinal en comparación con el grupo control no sólo a nivel de filo, sino también a nivel de clase y género, destacando una mayor abundancia relativa de los géneros *Lactobacillus* y *Blautia*. En este sentido, el género *Lactobacillus* se ha asociado ampliamente con el

desarrollo de obesidad y NAFLD[157,302,303], y el aumento de *Blautia* se ha relacionado con NAFLD en pacientes pediátricos[304].

Tras 3 semanas de intervención dietética y administración de *A. muciniphila* y quercetina, el simbiótico redujo la concentración de glucosa en sangre en ayunas, la concentración de insulina plasmática y el índice HOMA-IR observados a las 6 semanas de ingesta de HFD, destacando sus efectos beneficiosos para contrarrestar la resistencia a la insulina como ya había sido descrito previamente[243,251]. Además, se observó una reducción del tejido adiposo blanco y una mayor concentración plasmática de leptina tras la administración del posible simbiótico. En este sentido, la elevada concentración de leptina podría promover el gasto de energía y controlar la ingesta de alimentos para compensar los trastornos metabólicos causados por la dieta HFD[305].

A su vez, una reducción en la expresión génica relativa de Pparγ y *Plin2* en el tejido adiposo blanco se observó tras tres semanas de administración combinada de quercetina y *A. muciniphila*, lo que se ha asociado con un papel protector frente a la resistencia a la insulina y al desarrollo de obesidad y de NAFLD[306,307]. Además, la expresión hepática de genes involucrados en la lipogénesis *de novo* se incrementó con la dieta HFD, efecto que fue contrarrestado con la administración del posible simbiótico[308]. En cuanto al estado inflamatorio, la combinación de quercetina y *A. muciniphila* redujo significativamente la expresión relativa de citoquinas y marcadores proinflamatorios, mostrando un efecto protector del posible simbiótico en el estado inflamatorio, resultado que concuerda con estudios previos[243,251].

Los efectos beneficiosos del posible simbiótico también se reflejaron en la composición de la microbiota intestinal. A nivel de filo, la abundancia relativa del filo Cyanobacteria se incrementó tras tres semanas de suplementación, mientras que el filo Actinobacteria, un marcador común de obesidad[309], disminuyó significativamente. A nivel de género, *Blautia* (altamente detectado en pacientes adultos y pediátricos con NAFLD[304,310]) y *Coprobacillus* (anteriormente asociado con HFD[293]) se redujeron en todos los grupos experimentales suplementados con quercetina. Además, *Lactobacillus* y *Lactococcus*, ambos géneros correlacionados positivamente con la insulina plasmática en ayunas[310], redujeron su abundancia relativa tras la suplementación con quercetina y *A. muciniphila*. Todos estos resultados respaldan la capacidad del simbiótico en la remodelación de la microbiota intestinal y su efecto beneficioso para contrarrestar la obesidad y NAFLD.

Estos cambios en la composición de la microbiota intestinal causados por el posible simbiótico promovieron modificaciones beneficiosas en la composición, síntesis y transporte de ácidos biliares (BA) en el hígado. La intervención nutricional junto con la suplementación con *A. muciniphila* y quercetina aumentó la concentración plasmática total de ácidos biliares (BA, por sus siglas en inglés), principalmente BA hidrofílicos primarios y no conjugados, estableciendo un perfil de BA hidrofílico más saludable. Además, el aumento de la expresión de genes de síntesis y transporte de BAs sugirió una mejora en la circulación enterohepática asociada a la administración del simbiótico.

Finalmente, se llevó a cabo un análisis de correlación con estos resultados, mostrando una interconexión en la que el posible simbiótico parece inducir cambios en la microbiota intestinal, que a su vez están relacionados con la mejora observada durante en el desarrollo de obesidad y NAFLD en nuestro modelo *in vivo*.

En conclusión, la modulación del perfil de BAs, la regulación positiva de su flujo de síntesis y transporte, así como una modulación del estado inflamatorio hepático y el proceso de lipogénesis en el tejido adiposo blanco podrían denotar mecanismos subyacentes a los efectos beneficiosos del papel de *A. muciniphila* en la enfermedad metabólica y apoyar su uso en combinación con quercetina como un posible tratamiento del desarrollo de NAFLD y obesidad.

Tercera publicación

La disfunción mitocondrial, junto con el estrés oxidativo y la alteración de la microbiota intestinal constituyen factores clave que favorecen la progresión de NAFLD hacia esteatohepatitis no alcohólica (NASH, por sus siglas en inglés). De hecho, investigaciones recientes han revelado una posible interacción bidireccional entre la disfunción mitocondrial y la microbiota intestinal. Por tanto, centrar el estudio terapéutico en la disfunción mitocondrial podría suponer una estrategia para evitar la disrupción del eje intestino-hígado y así retrasar, o incluso prevenir, la progresión de NAFLD. En este sentido, la proteína MCJ es un regulador negativo endógeno de la cadena de transporte de electrones, y su inhibición incrementa la síntesis de ATP y limita la producción de estrés oxidativo, siendo capaz de frenar el desarrollo de NAFLD en modelos animales. A su vez, la deficiencia de MCJ modifica la relación existente entre la actividad mitocondrial y la microbiota intestinal. En base a ello, el objetivo de esta tercera investigación es determinar el efecto de la deficiencia de la proteína MCJ en un modelo murino de NASH, así como estudiar si los efectos observados asociados a dicha deficiencia pueden ser transferidos a ratones libres de gérmenes a través de un trasplante de microbiota cecal.

Para alcanzar dicho objetivo, ratones macho C57BL/6J de seis semanas de edad *wild type* (WT) y *knock-out* para la proteína MCJ (MCJ-KO) se alimentaron con una dieta control o una dieta rica en grasa y deficiente en colina y metionina (CDA-HFD, por sus siglas en inglés) durante seis semanas. Transcurrido este tiempo, los animales se eutanasiaron y las muestras se recogieron en las condiciones necesarias para su posterior uso y análisis. Un ratón de cada uno de los cuatro grupos experimentales de este primer modelo (modelo A) se seleccionó en base a parámetros relacionados con el desarrollo de NAFLD como donante de materia cecal. Así, ratones macho C57BL/6J libres de gérmenes se sometieron a un trasplante de microbiota cecal (TMC) y se dividieron en un total de ocho grupos experimentales en base a los cuatro tipos de donantes de materia cecal y en base a la dieta (C y CDA-HFD), constituyendo así el denominado modelo B. Tras tres semanas, los animales se sacrificaron y las muestras se recogieron en las condiciones necesarias para su posterior análisis.

En el modelo A, tras seis semanas de dieta CDA-HFD, los animales WT mostraron a nivel hepático la presencia de un alto grado de esteatosis, así como de inflamación y fibrosis, corroborando así la consecución del modelo dietético de NASH.

Por el contrario, se observó en el grupo MCJ-KO sometido a dicha dieta un menor estado inflamatorio y fibrótico, demostrando el papel protector de la deficiencia de la proteína MCJ en el desarrollo de NAFLD, tal y como se había indicado previamente en otros modelos animales[220]. A su vez, en relación con el estado intestinal, el grupo CDA-HFD/WT presentó un incremento de la permeabilidad, así como una alteración de la barrera intestinal y un consecuente estado de endotoxemia comparado con el grupo control, todo ello asociado con la progresión de NAFLD a NASH. En contraste, la deficiencia en la proteína MCJ en el grupo de ratones alimentados con la misma dieta mostró una mejora de estos parámetros, sugiriendo así que la actividad mitocondrial hepática derivada de la deficiencia en MCJ contrarresta la pérdida de la integridad de la barrera intestinal, reduce la translocación de productos bacterianos y modula la respuesta inflamatoria derivada de la progresión de NASH.

Debido al papel esencial que juega el microbioma intestinal en el desarrollo de NASH, se analizó la composición microbiana en heces fecales de este modelo A. Aunque la dieta CDA-HFD indujo un estado de disbiosis intestinal tanto a nivel de filo como a nivel de género, se identificó un perfil específico de microbiota intestinal asociado al genotipo MCJ-KO. Así, independientemente de la dieta, se observó un incremento de la abundancia relativa del género *Dorea*, así como una reducción de los géneros *AF12*, *Allobaculum* y [*Ruminococcus*]. En base a ello, el perfil de microbiota intestinal asociado al genotipo MCJ-KO podría ejercer un papel protector en el desarrollo de NASH, evitando la progresión de fibrosis y preservando la integridad de la barrera intestinal.

Para elucidar si el efecto protector asociado a la deficiencia en la proteína MCJ estaba mediado por la microbiota intestinal y, por tanto, podía ser transferido, se realizó el TMC a ratones libres de gérmenes. A nivel hepático, tres semanas de dieta CDA-HFD indujeron un estadio temprano de NAFLD, dado que se observó un incremento de la esteatosis hepática, junto con un estado inflamatorio incipiente. En este caso, los grupos de ratones libres de gérmenes alimentados con CDA-HFD y que habían sido trasplantados con microbiota procedente de ratones MCJ-KO presentaron un menor desarrollo de la enfermedad, demostrando así que el efecto hepatoprotector de la deficiencia en la proteína MCJ es transferible a través del TMC.

A su vez, el análisis de la composición de microbiota intestinal en este modelo B mostró efectos moduladores no sólo de la dieta, sino también del genotipo y dieta del donante. Así, el mismo perfil microbiano que se identificó en el modelo A asociado al

genotipo MCJ-KO se observó en el modelo B, indicando que esta huella microbiana es capaz de ser transferida mediante TMC. Además, la capacidad de la deficiencia de la proteína MCJ para contrarrestar la alteración de la barrera intestinal también se transfirió al modelo B.

La deficiencia en MCJ es capaz de incrementar la beta oxidación lipídica, así como de mejorar la esteatosis hepática en modelos animales de NAFLD[220]. Este hecho fue corroborado en nuestro modelo A, observándose además un incremento del metabolismo de NAD tanto a nivel hepático, como a nivel intestinal, tal y como se demostró con el aumento no solo de los niveles de NAD^+, sino también de las enzimas que participan en su síntesis. Estos hallazgos fueron reflejados en el modelo B, observándose tanto a nivel hepático como a nivel intestinal un incremento del metabolismo del NAD, así como de la beta oxidación lipídica en aquellos grupos alimentados con dieta CDA-HFD que habían sido trasplantados con microbiota procedente de MCJ-KO. Por tanto, el incremento en el metabolismo del NAD derivado de la deficiencia de la proteína MCJ puede ser transferido a través del TMC, dando lugar a un incremento de la oxidación hepática de ácidos grasos y reduciendo así la progresión de NAFLD.

Seguidamente, tras confirmar el perfil microbiano común en ambos modelos animales, se llevó a cabo el análisis del metaboloma fecal, así como el análisis de correlación para poder identificar posibles mecanismos a través de los cuales la microbiota intestinal pueda llevar a cabo los efectos protectores en el desarrollo de NAFLD. Los resultados mostraron que el metaboloma específico del genotipo MCJ-KO identificado en el modelo A no fue completamente transferido al modelo B, aunque algunos patrones, como pueden ser aquellos asociados a la adenosina o a la riboflavina, fueron identificados en ambos modelos y podrían estar participando en la mejora del metabolismo del NAD.

Por último, dado que los análisis de correlación apuntaban al género *Dorea* como uno de los taxones bacterianos que podrían estar involucrados en los efectos protectores observados, se llevó a cabo una validación de nuestros resultados con bases de datos humanas. Así, tras el análisis de datos disponibles de una cohorte pública de pacientes con diferentes grados de fibrosis, se observó que los pacientes con NAFLD que no padecían obesidad presentaban menor abundancia del género *Dorea* en comparación con

los pacientes con NAFLD y obesidad, sugiriendo que este género podría constituir un marcador vinculado a NAFLD no asociado a obesidad.

En conclusión, estos resultados respaldan que la deficiencia de MCJ y, en consecuencia, la potenciada actividad mitocondrial modulan un perfil microbiano específico y protector capaz de retrasar la progresión de la enfermedad en un modelo dietético agresivo de NASH, respaldando así la importancia de la relación mitocondria-microbiota en NASH y apuntan a nuevos enfoques terapéuticos.

Conclusiones

En base a los resultados obtenidos, así como a los objetivos previamente establecidos, las conclusiones que se deducen de la presente Tesis Doctoral son las siguientes:

» **Primera conclusión**

La cirugía bariátrica posee un efecto modulador a largo plazo sobre la composición y funcionalidad de la microbiota intestinal en pacientes con obesidad severa.

» **Segunda conclusión**

El perfil de microbiota intestinal resultante de la cirugía bariátrica después de cuatro años de la intervención está relacionado con una mejora del estado de salud de los pacientes, pudiendo ser una menor eficiencia en la extracción de energía de la dieta el posible mecanismo asociado a los efectos beneficiosos observados a largo plazo.

» **Tercera conclusión**

La combinación de una intervención nutricional junto con la administración del posible simbiótico formado por quercetina y *Akkermansia muciniphila* posee efectos beneficiosos que contribuyen a retrasar la progresión de la enfermedad en un modelo animal de obesidad temprana y NAFLD en rata.

» **Cuarta conclusión**

La modulación de la microbiota intestinal como consecuencia de la intervención nutricional y la administración de quercetina y *Akkermansia muciniphila* produce una regulación del metabolismo de los ácidos biliares, así como una modulación del estado inflamatorio hepático y del metabolismo lipídico en el tejido adiposo. Estas vías metabólicas podrían estar involucradas en la capacidad de esta estrategia combinada para contrarrestar el desarrollo de obesidad y NAFLD.

» **Quinta conclusión**

La deficiencia en la proteína MCJ confiere una actividad mitocondrial mejorada que es capaz de frenar la progresión de la enfermedad en un modelo murino dietético de NASH.

» **Sexta conclusión**

La deficiencia en la proteína MCJ es capaz de modular la composición y funcionalidad de la microbiota intestinal, resultando en un perfil microbiano específico, así como en una mejora del eje intestino-hígado y de la permeabilidad intestinal, lo que puede estar relacionado con los efectos beneficiosos observados en la progresión de NASH.

» **Séptima conclusión**

El perfil microbiano específico derivado de la deficiencia en la proteína MCJ es capaz de transferirse a ratones libres de gérmenes a través del trasplante de microbiota cecal, y ejercer efectos protectores que contribuyen a impedir la progresión de NASH.

› CONCLUSIÓN GENERAL ‹

La microbiota intestinal juega un papel esencial en la patogénesis y en el desarrollo de obesidad y NAFLD. Así, la modulación de la microbiota intestinal, no sólo como consecuencia de tratamientos actuales como la cirugía bariátrica, sino también a través de la relación mitocondria-microbiota o de otras estrategias como la administración de simbióticos, tiene importantes efectos beneficiosos en el desarrollo de estas enfermedades, siendo recomendable considerarlas como posibles alternativas terapéuticas. Sin embargo, tanto los mecanismos específicos como las vías de señalización a través de las cuales la modulación de la microbiota intestinal ejerce sus efectos beneficiosos siguen sin estar totalmente establecidos, por lo que profundizar en estos procesos es esencial para proponer tratamientos seguros, consistentes y efectivos que puedan diseñarse y combinarse en función de las características de cada paciente.

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