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Consequences of early life adverse events on the development of non-communicable diseases in mouse models

Hanna Ilchmann

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Hanna Ilchmann. Consequences of early life adverse events on the development of non-communicable diseases in mouse models. Veterinary medicine and animal Health. Institut National Polytechnique de Toulouse - INPT, 2019. English. NNT : 2019INPT0077 . tel-04168557

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Université
de Toulouse

THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

Institut National Polytechnique de Toulouse (Toulouse INP)

Discipline ou spécialité :

Pathologie, Toxicologie, Génétique et Nutrition

Présentée et soutenue par :

Mme HANNA ILCHMANN

le jeudi 19 septembre 2019

Titre :

Consequences of early life adverse events on the development of non-communicable diseases in mouse models

Ecole doctorale :

Sciences Ecologiques, Vétérinaires, Agronomiques et Bioingénieries (SEVAB)

Unité de recherche :

Toxicologie Alimentaire (ToxAlim)

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Mme SANDRINE MENARD, INRA TOULOUSE, Membre

Mme VASSILIA THEODOROU, EI PURPAN, Membre

THESE

En vue d l'obtention du

Doctorat de l'Université de Toulouse

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Discipline : Pathologie, Toxicologie, Génétique & Nutrition

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Le 19 septembre 2019

Conséquences d'événements adverses en période néonatale sur le développement de maladies non-transmissibles dans des modèles murins

Consequences of early life adverse events on the development of non-communicable diseases in mouse models.

Jury

Dr Philippe Gérard	Président du Jury
Dr Johanne Le Beyec-Le Bihan	Rapporteur
Dr Patricia Parnet	Rapporteur
Dr Michelle Kelly-Irving	Examineur
Dr Benoit Chassaing	Examineur
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Ecole doctorale : Sciences Ecologiques, Vétérinaires, Agronomiques, Bioingénieries

Unité de recherche : UMR 1331 INRA/INP/UPS Toxalim

Directeurs de thèse : Dr Sandrine Ménard et Dr Vassilia Theodorou

Meinem Ungeborenen...

For my unborn...

A mon enfant à naitre...

*For You formed my inward parts;
You wove me in my mother's womb.*

Psalm 139:13

REMERCIEMENTS

Je remercie **Dr Patricia Parnet** et **Dr Johanne Le Beyec-Le Bihan**, rapporteurs de ma thèse, d'avoir accepté d'évaluer le travail ici présenté. Je les remercie pour toute leur implication, le temps investi, les conseils, les encouragements, les remarques et critiques constructives.

Je remercie le président du jury **Dr Philippe Gérard** pour son investissement.

Merci aux examinateurs **Dr Michelle Kelly-Irving** et **Dr Benoit Chassaing**. Merci pour tous les échanges stimulants et passionnants. Merci d'avoir participé à l'aboutissement de ce travail. Merci **Michelle** pour les réunions stimulantes autour de la DOHaD. Tes présentations sont passionnantes. Merci **Benoit** pour les discussions autour de la barrière, du mucus, des peptides antimicrobiens. Merci pour ta bienveillance et compréhension.

Je remercie mes directeurs de thèse, **Pr Vassilia Théodorou** et **Dr Sandrine Ménard**.

Merci **Sandrine**, de m'avoir formé au métier du chercheur durant ces quatre dernières années. Merci d'avoir contribué à ce que je suis aujourd'hui dear #DoctorMother. Merci pour les nombreuses discussions scientifiques et personnelles. Merci de m'avoir transmis ta passion pour la Science. Merci de m'avoir toujours poussée à aller plus loin. Merci d'avoir cru en moi. Merci aussi pour ta compréhension et ta patience. Merci pour les heures et parfois les nuits passées à corriger mes nombreux rapports, dossiers, présentations, posters, candidatures, papiers, sans parler de ce manuscrit de thèse. Merci d'être pour moi un exemple de chercheuse déterminée, parfois têtue, créative, et visionnaire. Merci pour les voyages, les escapades culinaires diverses et variées et peut-être parfois excessives. Merci pour les larmes de rire. Ah ! mes muscles abdominaux ne se sont pas dégradés !

Merci **Vassi** de m'avoir acceptée et bien accueillie dans ton équipe. Merci pour tout ton investissement dans ma thèse. Merci pour les échanges stimulants autour de mes résultats. Tes commentaires pour améliorer mes papiers, les remarques critiques qui me poussaient de quitter ma zone de confort. Merci de m'avoir poussée aller plus loin. Merci d'avoir toujours cru en moi et de m'avoir soutenue dans mes projets et ambitions. Merci pour le calme que tu gardes et la bienveillance que tu dégages. Merci pour ce petit côté maternel.

Je remercie les membres de mon comité de thèse qui m'ont accompagné durant ces trois dernières années. Merci au **Dr Valérie Verhasselt**, **Dr Pierre Gourdy** et **Dr Laurence Guzylack-Piriou**.

Merci **Dr Valérie Verhasselt** pour toutes les idées partagées. Merci pour la considération que tu exprimais pour notre travail. Merci de partager d'avoir partagé ta passion et motivation.

Je remercie **Dr Pierre Gourdy** pour ses remarques ouvrant mon horizon. Merci pour l'œil critique de clinicien sur ce travail de thèse. Merci pour l'analyse claire et l'orientation utile.

Je remercie **Dr Laurence Guzylack-Piriou** pour tout son aide, ses sacrifices et son implication dans ma thèse. Merci pour tes encouragements, tes idées, ton œil critique, les nombreuses explications. Merci pour les retours constructifs sur mes dossiers et papiers. Merci pour toutes les questions que tu me posais lors de mes répétitions.

Je remercie les différents collaborateurs avec qui j'ai eu la joie de travailler dans le cadre de ma thèse.

Merci au **Dr Maiwenn Olier** pour toutes les analyses du microbiote. Merci pour les nombreuses explications. Merci pour tout le temps investi dans nos manip. Merci pour tes encouragements, tes nombreuses relectures, la joie et le calme que tu dégages. Merci pour ta rigueur – combien j'aimerais être comme toi ! Merci pour les fous rires et d'avoir eu assez de clairvoyance pour prendre des photos du siphon pété !!!

Merci **Corinne Lencina** pour ton implication dans ma thèse. Merci pour toute ton aide pour nos manip. Merci pour toutes les heures investies à me former à des ELISAs. Merci pour la belle collaboration en salle de bioch. J'ai bien aimé nos jours de challenge où on passait 6 plaques ELISA à la fois. Merci pour les bons moments passés ensemble, à la couture, pendant les repas, entre les manip, dans les pauses café, et je ne mentionne pas tout... Merci pour tes gâteries culinaires et ta créativité. Merci d'être la colle pour éviter que le bateau se brise. Merci d'avoir toujours pris soin de moi et de mon bien-être.

Merci à **Dr Sandrine Ellero-Simatos**, **Dr Hervé Guillou**, **Sharon Barretto** et **Céline Lukowicz** pour vos remarques sur le volet métabolisme du projet Metalogin. Merci **Sandrine** pour la sortie en vélo pour rejoindre INSA, les manip ensemble, le temps investi pour me

former, les nombreux commentaires sur mon travail, les conseils. Merci pour ta bienveillance et les petits échanges dans le couloir. Merci **Hervé** pour ton soutien, ta considération pour notre travail, tes remarques, ton aide dans la préparation de ma thèse. Merci pour ta gentillesse, tes encouragements.

Merci à **Elia Macadré**, stagiaire pendant quatre mois avec nous. Merci pour ton travail énorme, ta motivation, ta disponibilité, ton envie d'apprendre, ta patience avec moi. Ce fut une belle expérience avec toi !

Merci à **Dr Colette Denis** et **Dr Marie Buléon** pour la collaboration dans le projet NOD. Merci pour vos remarques pertinentes, vos disponibilité et motivation.

Merci à **Dr Julien Diana** pour les échanges autour du projet « MS and type 1 diabetes ». Merci pour la considération, les conseils et la collaboration.

Merci à **Dr Sonia Lamandé** pour les beaux échanges autour de l'immunité dans la petite enfance. Merci d'avoir accueilli chez toi nos animaux.

Merci à **Valérie Bacquié** pour ta patience à me former à l'immunohistomarquage.

Merci au **Pr Jamileh Movassat** de m'avoir accueillie pour un stage dans son équipe à Paris (BFA, B2PE, Paris Diderot). Merci **Junjun Liu** de m'avoir formée avec beaucoup de soin. Merci à **Blandine** et **Pengfei** pour les bons moments passés ensemble.

Je remercie **Thierry Gauthier** du plateau M2C. Merci pour tes patience et pédagogie pour me former à la cytométrie et au microscope confocale. J'ai véritablement apprécié ces formations. Tu as été très didactique !

Un grand merci à **Christelle Cartier** pour toute ton aide et ses conseils autour de l'immunohistomarquage. Merci pour ta patience, ta réactivité, tes conseils pratiques. Merci aussi pour ta gentillesse, ton sourire, les échanges, les temps passés ensemble à la couture.

Je remercie **Dr Rémy Burcelin** pour ses remarques stimulantes concernant ce projet de thèse. Merci de m'avoir acceptée il y a 7 ans pour mon premier stage en laboratoire de recherche – ça a été une révélation pour moi.

Merci **Fabrice Pierre** pour ta toujours bonne humeur et bienveillance, ta considération et tes encouragements.

Merci aux autres thésards dans le couloir : Merci à **Yann Malaisé, Sophie Yvon, Jasper Kamphuis, Kévin Gillois et Anaïs Mazenc**. Merci **Yann** pour tous les conseils pendant ma première année de thèse, tes encouragements, les pauses ensemble, les fous rires – je ne vais jamais oublier notre aventure « Hanovre » ! Merci **Sophie** pour toutes les occasions où tu m’as bien fait rire, ta comédie, tes conseils. Merci **Jasper** d’avoir été un renfort de la culture germanique à Toxalim. J’ai bien apprécié nos petits débats sur tout et rien ! Merci pour ton aide pour améliorer mon anglais, perfectionner mes diapos et mon manuscrit.

Merci à tous ceux de l’équipe NGN que je n’ai pas encore mentionnés : **Cathy, Christine, Hervé, Hélène, Muriel, Valérie Be, Valérie M et Valérie T**, ainsi que les anciens membres de NGN: **Kheira, Michèle, Alain, Afifa, Laurent**. Merci pour votre soutien, l’intérêt que vous avez pour moi, votre participation à mes nombreuses répétitions, vos remarques, suggestions, vos questions pour me préparer aux différents oraux.

Je remercie les membres du Journal Club. Même si le plaisir fut court, je me suis beaucoup réjouie de nos petites heures passées ensemble: **Benoit, Manon, Yann, Jasper**.

Merci à l’équipe d’animalerie : **Caroline, Colette, Elodie, Géraldine et Mickael**. Merci **Caroline** pour la gestion de nos animaux, ton aide, tes idées et ton implication dans nos manips.

Je remercie **Christian Chervin et Benoit van der Rest** pour les bons moments partagés lors des enseignements à l’école d’ingénieur ENSAT. J’ai appris plein de choses intéressantes !

Merci aux **ADASSiens dans l’activité couture et jardin**. Merci pour les bons moments partagés ensemble. Merci **Joelle et Claire** d’avoir organisé ces activités.

Je remercie les gestionnaires de Toxalim: **Sarah Calcagno, Marie Hélène Piquereau**. Merci de rendre notre travail possible. Merci de votre réactivité, les réponses aux questions, votre gentillesse.

Merci **Jeannette Faramond** pour ton accueil ! C’était toujours un plaisir d’être accueillie par toi. Merci pour toute orientation et aide dans les démarches administratives. Merci pour le temps passé ensemble dans la pause de midi, les bons échanges.

Merci à la RH de l’INP **Marie-Claude Portell**. Merci pour votre réactivité, aide et orientation.

Je remercie **Dominique Pantalacci** pour ses mails prompts et toujours plein de gentillesse, même lorsque moi j'étais à la bourre.

Je ne souhaite pas oublier mes amis et ma famille qui m'ont supportée durant ces dernières années :

Merci à l'armée de prière qui s'est tenue derrière moi pendant tous ces derniers trois ans et en particulier pendant le temps de rédaction : merci **Honorine**, merci **Lois**, **Fanja**, **Citra**, **Michel**, **Johnny**, et tous les autres qui se sont tenus derrière moi.

Merci **Rhoda** pour ton écoute, les échanges, tes conseils, tes encouragements. Merci **Nathalie** pour tous les échanges, les encouragements et les discussions. Ce fut un plaisir de rencontrer des scientifiques à l'EPET. J'en remercie notre Seigneur !

Je tiens aussi à remercier les membres de l'association des groupes bibliques universitaires – **GBU**. Merci pour les bons moments passés ensemble pendant les discussions autour de la Bible. Un merci particulier à **Caroline** pour tout ton soutien et tes prières. Merci au **réseau GBU doctorants**. Les échanges avec vous ont été très enrichissants. Ça m'a fait tellement du bien de me retrouver avec vous une fois par an.

Ich möchte auch von Herzen meiner Familie, insbesondere meinen Eltern danken. Danke **Mama** und **Papa** für all eure Unterstützung und Ermutigung, seit ihr mich auf der Welt willkommen geheißen habt. Danke, dass ihr immer in meinen Projekten voll und ganz hinter mir standet, mich nicht verunsichert, sondern immer ermutigt habt. Danke, dass ihr an mich glaubt und mir beigebracht habt auf meinen eigenen Beinen zu stehen. Danke Mama, für deine Fürsorge und Treue. Danke Papa für die Ruhe die du ausstrahlst.

Danke **Ruth** für alle Unterstützung in meiner Schul- und Studienlaufbahn. Danke für das viele Korrekturlesen, die Diskussionen, Tipps zum Verbessern, deine Ermutigungen ... Danke, dass du so eine tolle Schwester bist. Danke **Uli** für deine lieben Worte der Ermutigung.

Danke **Joel** und **Friederike** für eure Liebe. Danke **Andi** und **Lydi**, dass ihr mich so oft spüren habt lassen, dass ihr stolz auf mich seid.

Danke **Oma** für deine treuen Gebete. Danke **Judith**, dass du mir immer das Gefühl gegeben hast, dass du meine Meinung und meine wissenschaftliche Laufbahn schätzt.

Last but not least, je remercie **mon cher époux Bailly Franck Eric Diounou**. Merci d'être mon compagnon dans cette course sur la terre. Merci de m'avoir accompagnée tout au long de ces dernières années de master et thèse. Merci pour ton écoute. Merci pour ton soutien pratique, psychologique et émotionnel. Merci de m'avoir toujours soutenue dans mes ambitions et ne jamais m'avoir découragée. Merci de m'avoir bien souvent ouvert les yeux sur la réalité quand j'avais la tête dans le guidon. Merci d'avoir toujours orienté ma vue vers celui qui nous connaît véritablement et récompense justement. Merci pour ton amour. Je t'aime.

ORIGINAL ARTICLES

Ilchmann-Diounou H, Olier M, Lencina C, Riba A, Barretto S, Nankap M, Sommer C, Guillou H, Ellero-Simatos S, Guzylack L, Theodorou V, Ménard S. Early life stress induces type 2 diabetes-like features in aging mice. *Brain Behavior and Immunity* 2019 <https://doi.org/10.1016/j.bbi.2019.04.025>

Ilchmann-Diounou H, Buléon M, Bacquié V, Theodorou V, Denis C, Ménard S. Non-Obese Diabetic Mouse Model is emphasizing divergence between *in vivo* and *ex vivo* intestinal permeability measurements. *Submitted to Scientific Reports 23 July 2019, under review.*

REVIEW ARTICLE

Ilchmann-Diounou H and Ménard S. Psychological Stress, Intestinal Barrier Dysfunctions and Autoimmune Disorders. *Submitted to Frontiers in Immunology 17 May 2019, under review.*

ORAL COMMUNICATIONS

Ilchmann-Diounou H, Buléon M, Bacquié V, Theodorou V, Denis C, Ménard S. Comparaison des mesures de perméabilité intestinale *in vivo* et *ex vivo* chez la souris NOD (Non Obese Diabetic) (2019). Réunion annuelle du Groupe Français de Neuro-Gastroenterologie, Toulouse, France

Ilchmann-Diounou H, Buléon M, Bacquié V, Theodorou V, Denis C, Ménard S. Comparaison des mesures de perméabilité intestinale *in vivo* et *ex vivo* chez la souris NOD (Non Obese Diabetic) (2019). Club d'études des cellules épithéliales digestives XXXVIIème réunion, Toulouse, France

Ilchmann H, Olier M, Lencina C, Riba A, Barretto S, Nankap M, Sommer C, Guillou H, Ellero-Simatos S, Guzylack L, Theodorou V, Ménard S. Le stress de séparation maternelle induit chez l'adulte les symptômes semblables du diabète de type 2 associés à un défaut de sécrétion d'IL-17 et IL-22 (2018). 4ème congrès de la SF-DOHaD, Grenoble, France

Ilchmann H, Olier M, Lencina C, Ellero-Simatos S, Nankap M, Riba A, Sommer C, Guzylack-Piriou L, Théodorou V, Ménard S (2018). Early life stress in mice induces type 2 diabetes-like symptoms associated with defect of intestinal IL-17 and IL-22 secretion at

adulthood. Club d'études des cellules épithéliales digestives XXXVIème réunion, Marseille, France

Ilchmann H, Olier M, Lencina C, Ellero-Simatos S, Nankap M, Riba A, Sommer C, Guzylack-Piriou L, Théodorou V, Ménard S (2017). Early life stress induces type 2 diabetes-like symptoms at adulthood in mice associated with defect of intestinal IL-17 and IL-22 secretion. Microbes, Immunity and Metabolism International Conference, Paris, France

Ilchmann H, Olier M, Lencina C, Ellero-Simatos S, Nankap M, Riba A, Sommer C, Guzylack-Piriou L, Théodorou V, Ménard S (2017). Early life stress induces type 2 diabetes-like symptoms at adulthood in mice associated with defect of intestinal IL-17 and IL-22 secretion. 10th International Meeting of Pediatric Endocrinology, Washington DC, USA

Ilchmann H, Lencina C, Ellero-Simatos S, Riba A, Harkat C, Sommer C, Guillou H, Guzylack-Piriou L, Olier M, Théodorou V, Ménard S (2016). Chronic maternal separation in mice impairs immuno-metabolism in older offspring on standard diet. The Neonatal Window of Opportunity – Early Priming for Life, Hanovre, Germany

POSTERS

Ilchmann-Diounou H, Olier M, Lencina C, Riba A, Barretto S, Nankap M, Sommer C, Guillou H, Ellero-Simatos S, Guzylack L, Theodorou V, Ménard S (2019). Early life stress induces type 2 diabetes-like features in aging mice. NeuroGASTRO 2019, Lisbon, Portugal.

Ilchmann H, Olier M, Lencina C, Ellero-Simatos S, Nankap M, Riba A, Sommer C, Guzylack-Piriou L, Théodorou V, Ménard S (2018). Early life stress induces type 2 diabetes-like symptoms at adulthood in mice associated with defect of intestinal IL-17 and IL-22 secretion. Modelling the Mammalian-Microbiota Host Superorganism, Paris, France.

Ilchmann H, Olier M, Lencina C, Ellero-Simatos S, Nankap M, Riba A, Sommer C, Guzylack-Piriou L, Théodorou V, Ménard S (2018). Early life stress induces type 2 diabetes-like symptoms at adulthood in mice associated with defect of intestinal IL-17 and IL-22 secretion. Mucosal Immunology Course and Symposium, Oxford, UK.

Ilchmann H, Olier M, Lencina C, Ellero-Simatos S, Nankap M, Riba A, Sommer C, Guzylack-Piriou L, Théodorou V, Ménard S (2018). Early life stress induces type 2 diabetes-like symptoms at adulthood in mice associated with defect of intestinal IL-17 and IL-22 secretion. Digestive Disease Week, Washington DC, USA.

Ilchmann H, Lencina C, Riba A, Sommer C, Guzylack-Piriou L, Olier M, Théodorou V, Ménard S (2017). Early life stress induces glucose intolerance and insulin secretion failure in aging female mice. 10th International Meeting of Pediatric Endocrinology, Washington DC, USA.

Ilchmann H, Lencina C, Ellero-Simatos S, Riba A, Harkat C, Sommer C, Guillou H, Guzylack-Piriou L, Olier M, Théodorou V, Ménard S (2016). Chronic maternal separation in mice impairs immuno-metabolism in older offspring on standard diet. The Neonatal Window of Opportunity – Early Priming for Life, Hannover, Germany

PRICES AND GRANTS

- 2019 **2nd prize of nEUROgastroTANDEM meeting 2019** in Lisbon, Portugal
- 2019 **Prix Claude Rozé**, Club d'études des cellules épithéliales digestives
- 2018 **Best oral communication** 4th congress of SF-DOHaD, Grenoble, France
- 2017 **Early Career Scientific Development Grant** for a two-month research mobility at Paris Diderot financed by the European Society of Pediatric Endocrinology (ESPE)
- 2017 **Travel Grant** for the 10th International Meeting of Pediatric Endocrinology financed by the European Society of Pediatric Endocrinology (ESPE)
- 2016 **Travel Grant** for The Neonatal Window of Opportunity,– Early Priming for Life, Hannover, Germany financed by Volkswagenstiftung

TEACHING EXPERIENCE DURING PHD

DCCE 2016-2019 at Toulouse INP - Ecole National Supérieur Agronomique de Toulouse (ENSAT). Tutor activity in the following courses under the supervision of Christian Chervin, Benoit van der Rest and Thierry Liboz.

Food Science – De la vigne au vin (1^{ère} année ingénieur)

Food Science – Cuisine moléculaire (2^{ème} année ingénieur)

Food Science – Analyse sensorielle (2^{ème} année ingénieur)

Enzymologie (classe préparatoire)

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ABBREVIATIONS

5-HT	Serotonin
ACTH	adrenocorticotrophic hormone
AD	autoimmune disorder
AMP	antimicrobial peptides
ANS	autonomous nervous system
BMI	body mass index
CLP	common lymphoid progenitor
CNS	central nervous system
CRAMP	cathelin-related antimicrobial peptide
CRF	corticotropin releasing factor
CRP	c-reactive protein
CS	caesarian section
DALYS	disability adjusted life years
DC	dendritic cell
DNA	deoxyribonucleic acid
DOHaD	Developmental Origins of Health and Disease
DSS	dextran sulfate sodium
dsRNA	double-stranded ribonucleic acid
EAE	experimental autoimmune encephalomyelitis
ENS	enteric nervous system
FcRn	neonatal Fc receptor
FD4	FITC-Dextran 4 kDa
FSS	fluorescein sodium salt
GALT	gut-associated lymphoid tissue
GAP	goblet-cell associated passage
GF	germ-free
GFP	green fluorescent protein
GIP	glucose-dependent insulinotropic polypeptide
GLP-1	glucagon-like peptide-1
GM-CSF	granulocyte macrophage colony stimulating factor
GR	glucocorticoid receptor
HFD	high-fat diet
HFHSD	high-fat high-sugar diet

HOMA	homeostatic model assessment of insulin resistance
HPA	hypothalamic pituitary adrenal axis
HRP	horse radish peroxidase
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IFN γ	interferon γ
Ig	immunoglobulin
iHMP	integrative human microbiome project
iIEL	intestinal intraepithelial lymphocytes
IL	interleukin
ILC	innate lymphoid cell
ILF	isolated lymphoid follicles
IQ	intelligence quotient
IRS-1	insulin receptor substrate-1
LP	<i>lamina propria</i>
LPS	lipopolysaccharide
LTi	lymphoid tissue inducer
M cell	microfold cell
MAMP	microbe associated molecular pattern
MHC	major histocompatibility complex
MLC	myosin light chain
MLCK	myosin light chain kinase
MLN	mesenteric lymph node
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MS	maternal separation
NK	natural killer
NOD	nucleotide-binding oligomerization domain
PND	post-natal day
PP	Peyer's patch
PRR	pattern recognition receptor
PTSD	post-traumatic stress disorder
qPCR	quantitative polymerase chain reaction
SCFA	short chain fatty acid
sIgA	secretory immunoglobulin A

siLP	small intestine lamina propria
SNS	sympathetic nervous system
sPLA2	phospholipase-A2
ssRNA	single-stranded ribonucleic acid
T1D	type 1 diabetes
T2D	type 2 diabetes
Th	T helper
TJ	tight junction
TLR	toll-like receptor
TNF α	tumor necrosis factor α
Treg	regulatory T cell

CONTENT

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RESUME

Le concept des origines développementales des maladies et de la santé (DOHaD) émet l'hypothèse d'une origine périnatale des maladies non-transmissibles (NCD). Le modèle de stress de séparation maternelle (MS) est largement utilisé chez le rongeur comme un paradigme d'événements adverses en période néonatale. Mon projet de doctorat, a eu pour but d'étudier les effets à long-terme du MS sur les fonctions de barrière intestinale, le métabolisme, la réponse immunitaire, l'auto-immunité ainsi que sur le microbiote, chez des souris mâles et femelles sauvages âgées sous régime standard. Le but étant de fournir des données expérimentales soutenant le lien entre stress néonatal et développement de désordres métaboliques ou auto-immuns à l'âge adulte.

Dans une première étude, nous avons montré que, le MS a induit une intolérance au glucose et une perte de la sensibilité à l'insuline associée à une dysbiose fécale chez des souris mâles sauvages C3H/HeN âgées de 350 jours (PND350). Le MS a diminué les concentrations d'IgG fécales et a augmenté les IgG anti-*E. coli* plasmatiques, représentant la réponse humorale contre le microbiote commensal. Le MS a diminué significativement la sécrétion d'IL-17 et IL-22 en réponse à une stimulation du TcR et a augmenté la sécrétion de TNF α en réponse à une stimulation LPS dans une culture de cellules de la *lamina propria* de l'intestin grêle (siLP). Les mêmes résultats ont été obtenus au niveau systémique (rate). Nous avons ainsi démontré pour la première fois que le stress néonatal est un facteur de risque pour le développement des désordres métaboliques chez la souris sauvage âgée sous régime standard. Nous avons écarté un rôle exclusif du microbiote dans l'intolérance au glucose induite par le MS avec des expérimentations de transfert de microbiote fécal.

Dans une seconde étude, chez les femelles PND350 soumises au MS, on a observé une augmentation de la sécrétion d'IL-17 et IL-22 en réponse à une stimulation du TcR, et de TNF α avec ou sans stimulation au LPS par les cellules de la siLP. Nous avons observé en plus une inflammation systémique. Les souris MS ont développé une intolérance au glucose associée à une baisse de la sécrétion de l'insuline en réponse à un challenge au glucose. Le ratio de la surface des cellules β sur la surface du pancréas a légèrement diminué chez les MS et la valeur de ce ratio a corrélé positivement avec la sécrétion d'insuline induite par le glucose. En somme, le MS induit chez les souris femelles des effets à long terme sur l'immuno-métabolisme et l'homéostasie du pancréas.

Enfin, nous avons comparé les mesures de perméabilité intestinale *in vivo* (gavage) et *ex vivo* (chambres de Ussing) avec du FITC-Dextran 4 kDa dans un modèle de diabète de type 1 (NOD - souris non-obese diabetic). De façon inattendue, les résultats ont différencié en fonction des méthodes et cette différence n'est pas due à un défaut de la fonction rénale induite par le diabète. Par contre, nous avons observé un allongement de l'intestin grêle chez les souris diabétiques qui a corrélé positivement avec la perméabilité intestinale *in vivo*. Le diabète n'a pas modifié le transit intestinal, l'humidité des fèces et l'apparence histologique de l'intestin. En somme, nos résultats soulignent l'importance de distinguer la perméabilité intestinale, exprimée en cm/s mesurée *ex vivo*, et la notion d'exposition systémique aux antigènes luminaux mesurée *in vivo*.

Mon travail de thèse montre que les événements adverses néonataux sont un facteur de risque pour les NCD. Il est intéressant de noter que nos observations sur les souris âgées sont similaires aux observations épidémiologiques. En effet, nos résultats préliminaires suggèrent que les souris MS femelles développent des désordres métaboliques avec des caractéristiques auto-immunes; alors que les mâles développent des désordres métaboliques plus classiques : résistance à l'insuline. Mon travail sur le modèle MS souligne l'importance de la vie néo-natale dans l'établissement de l'homéostasie et conforte le concept de DOHaD.

Stress social, Métabolisme glucidique, Barrière intestinale, Réponse immunitaire, Origines développementales des maladies et de la santé.

SUMMARY

The concept of Developmental Origins of Health and Disease (DOHaD) highlights the importance of early life period and raises the hypothesis that Non Communicable Diseases (NCD) could find their origins in perinatal environment. Neonatal maternal separation (MS) is a stress model widely used in rodents as a paradigm of early life adverse events. In my PhD project, I aimed to investigate in aging male and female wild-type mice under normal diet the long-term effects of neonatal MS on intestinal barrier function, metabolism, immunity, autoimmunity, as well as on microbiota. My work aimed to provide experimental data to support a link between early life stress and development of metabolic or autoimmune disorders with aging.

In our first study, MS led to glucose intolerance and loss of insulin sensitivity associated with fecal dysbiosis in Post Natal Day (PND) 350 wild-type C3H/HeN male mice fed a standard diet. Fecal IgG concentrations were decreased in MS mice compared to control mice, whereas anti-*E. coli* IgG, representing humoral response toward commensal microbiota, were significantly increased in plasma of MS mice. MS significantly decreased IL-17 and IL-22 secretion in response to TcR stimulation in small intestine *lamina propria* (siLP) culture. Besides, TNF α secretion in response to LPS-stimulation was slightly increased. The same results were obtained at systemic level (spleen). For the first time, we demonstrated that early life stress alone is a risk factor for metabolic disorders development in aging wild type mice under normal diet. The result of this project gave us the opportunity to question the role of microbiota in MS-induced glucose intolerance. Fecal microbiota transfer of MS mice microbiota was not sufficient to induce glucose intolerance.

In our second study in PND350 female, MS increased IL-17 and IL-22 by siLP cells in response to TcR stimulation. TNF α secretion with and without LPS stimulation was also increased by MS. Additionally, we observed systemic low-grade inflammation. MS mice developed glucose intolerance associated with decreased insulin secretion in response to glucose stimulus. Ratio of β -cell surface to pancreas surface was slightly decreased in MS mice compared to control. This ratio positively correlated with insulin secretion induced by glucose. Taken together, the results of our study showed that MS in wild type female mice under normal diet leaves a long-lasting imprinting on immune-metabolism and pancreas homeostasis.

We compared *in vivo* and *ex vivo* intestinal permeability measurements in a model of type 1 diabetes (NOD – non-obese diabetic mice). Intestinal permeability was assessed *in vivo*

by gavage and *ex vivo* in Ussing chambers with the marker FITC-Dextran 4 kDa. Surprisingly, the results of both methods were divergent. The difference between *in vivo* and *ex vivo* measurements could not be explained by altered renal excretion. Curiously, diabetic NOD mice had significantly longer small intestine than non-diabetic NOD mice and small intestine length positively correlated with intestinal permeability *in vivo*. However, there were no difference in intestinal transit time, feces humidity and histological appearance. Altogether, our results highlighted the importance to distinguish intestinal permeability, which is expressed as cm/s measured *ex vivo*, and the notion of systemic exposition to luminal antigen measured *in vivo*.

My PhD project shows that early life adverse events are a risk factor for NCD. Interestingly, our observations in aging mice are similar to epidemiological observations. Indeed, preliminary results suggested that female MS mice develop metabolic disorders with autoimmune characteristics but male MS mice develop classical metabolic disorders with insulin resistance. My work in MS model highlights the importance of early life in the establishment of homeostasis and comforts the concept of DOHaD.

Social stress, Glucose metabolism, Intestinal barrier, Immune response, Developmental origin of health and diseases (DOHaD).

INTRODUCTION

The incidence of non-communicable diseases (NCDs) is constantly increasing in the last decades. Among NCDs, there are metabolic disorders, like obesity and type 2 diabetes, autoimmune diseases, as for example type 1 diabetes and allergies. Explications for the important rise in NCDs are numerous and diverse. There are for example, changes in lifestyle, nutrition, increased exposure to environmental contaminations and pollutants,

Early life is an important period for the establishment of lifelong beneficial homeostasis between the organism and its environment, especially its microbiota. The concept of developmental origins of health and disease (DOHaD) suggests that early life can have potent imprinting mechanism on host's physiology and that adverse perinatal physical and social environment can lead to higher susceptibility to NCDs. Indeed, the thousand first days of our life, from conception to our second year's birthday, are described to be decisive for future health.

The intestine is the body's greatest surface in contact with its environment. In order to protect the organism from harmful substances and microorganisms but maintaining at the same time efficient nutrient absorption, the intestinal barrier is highly developed and regulated. Principal actors of the intestinal barrier are microbiota, intestinal epithelium with its highly regulated permeability and intestinal immune system. Interestingly, a tremendous amount of studies has shown that disturbed microbiota homeostasis (dysbiosis) is associated with a multitude of diseases, especially NCDs. Indeed, the gastro-intestinal tract seems to play a crucial role in organism's health and disease.

This work is the continuation of previous studies performed in our laboratory, which highlighted the impact of stress on the integrity of the intestinal barrier. Especially early life stress has been of particular interest in our laboratory. Indeed, early life stress is known to impair intestinal barrier through induction of intestinal hyperpermeability, low-grade inflammation and microbiota dysbiosis in young rodents. Maternal separation (MS) is a paradigm of early life stress in rodents.

During my PhD I had the opportunity to write a review article, submitted and under review for the journal *Frontiers in Immunology*. Some parts of this article are quoted in I. Part: State of the Art.

**I. PART
STATE OF THE ART**



CHAPTER I

INTESTINAL BARRIER AND INTESTINAL HOMOEOSTASIS

The human intestine is about six meters long and due to multiple folding through crypts of Lieberkühn, villi and microvilli, the surface is about 400 m². This makes the intestinal epithelium the mammalian organism's biggest surface in contact with the environment (**Figure 1**). A principal role of intestine is to complete the digestion and absorb the nutrients but at the same time, the intestine filters and defends the organism from harmful luminal content (pathogens, toxins...), while maintaining tolerance towards commensal microbiota and food antigens. Hence, the intestine is acting as a selective barrier. Considering the challenging, various, decisive and conflicting roles, it is not surprising that intestinal barrier function is highly diverse and well developed.

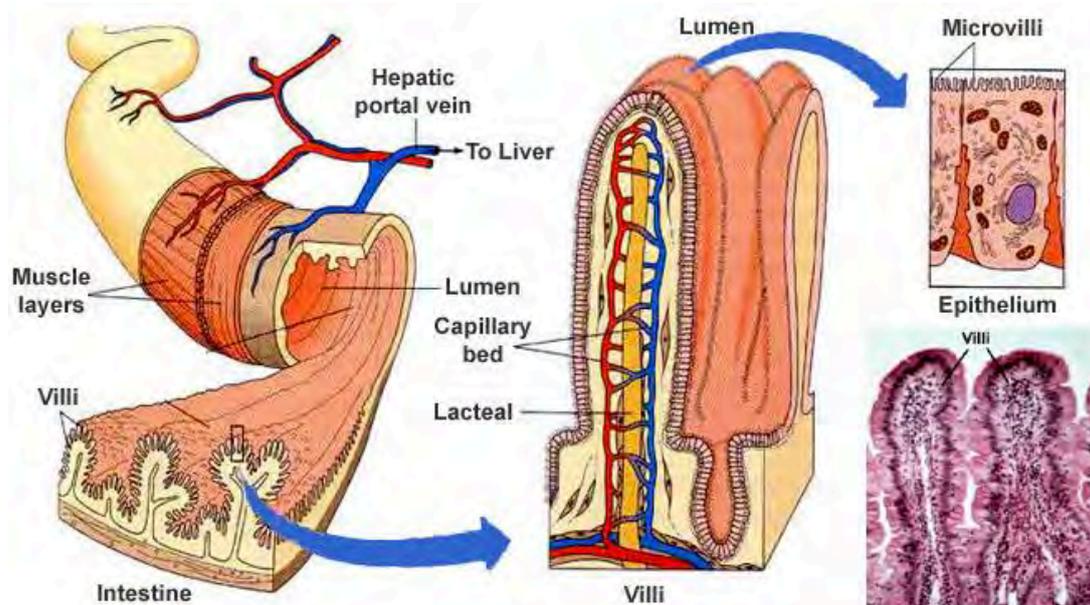


Figure 1 Intestinal anatomy. The intestinal tube is surrounded by two muscle layers, the longitudinal outer and the circular inner layer. The mucosal surface is increased by circular folds. Villi are finger-like extensions of mucosa into the intestinal lumen increasing further the intestinal surface. Intestinal epithelial cells have microvilli on apical site, which are also increasing the intestinal surface.

(<https://histaminefriendlykitchen.com/wp-content/uploads/2017/08/TheVilliandmicrovilli.jpg>)

1. Actors of intestinal barrier

Among the main actors of intestinal barrier, there are intestinal microbiota, mucus, intestinal epithelium, intestinal immune system with its innate and adaptive response, harbored within the *lamina propria* (LP), and muscular layer, responsible for gut motility.

1.1 Intestinal microbiota

The intestinal microbiota is a complex bacterial community colonizing the gut. There are more than 100 trillions of microorganisms from two major phyla, Bacteroidetes and

Firmicutes, representing around 90% of gut microbiota (Rinninella et al., 2019). Other represented phyla are for example Actinobacteria and Proteobacteria. Colonization density is increasing from stomach (10^1 - 10^3 bacteria/g), via duodenum (10^4 - 10^5 bacteria/g) and jejunum/ileum (10^8 bacteria/g) to colon (10^{12} - 10^{14} bacteria/g) (Nicolas, 2013). Microbiota plays an important role by providing, extracting and absorbing various nutritional compounds and metabolites, as for example bile acids, amino acids, vitamins, lipids, short-chain fatty acids (SCFAs) (Jandhyala et al., 2015). Lactate, as an example for a bacterial metabolite, has stimulating effect on colonic proliferation (Okada et al., 2013). Also small intestinal stem cells are using lactate as an energy source (Rodríguez-Colman et al., 2017). Microbiota can also influence host's physiology through its metabolites. For example, butyrate, a SCFA produced by gut microbiota, is able to regulate host metabolism and immunity *via* its utilization as energy source through β -oxidation in enterocytes and inhibition of histone deacetylases, affecting host's gene expression (Stilling et al., 2016). As an example, in cell cultures of murine mast cells and primary bone marrow mononuclear cells, butyrate has been shown to suppress proliferation and the production of the cytokines IL-6 and TNF α via the inhibition of histone deacetylase (Zhang et al., 2016). Microbiota participates also in the protection against pathogens colonization by occupation of ecological niches (competition for nutrients and space) (Jandhyala et al., 2015). Additionally, microbiota contributes to maturation of intestinal epithelium and immune system. This fact particularly strikingly visible in germ-free (GF) animals, which are living in sterile isolators, devoid of any microbiota. Indeed, GF mice are described to have thinner small intestine *lamina propria* (siLP) (Round and Mazmanian, 2009) and a great deficit in gut associated lymphoid tissue, as small Peyer's patches containing only a little number of germinal centers (Macpherson et al., 2001). They have immature immune system, namely reduced number of IgA producing and CD4⁺ cells. Colonization by microbiota can restore these defects (Falk et al., 1998; Helgeland et al., 1996; Macpherson et al., 2001).

1.1.1 Microbiota during life-time

Microbiota is depending on lots of different factors and can evolve during lifetime. *In utero* the fetus is considered sterile (Hornef and Penders, 2017), even if this assumption is debated. Some affirm that there is a microbiota *in utero* (Aagaard et al., 2014; Jiménez et al., 2008). However, the presence of bacteria is often confirmed by qPCR and rarely by culture of living bacteria and critics are highlighting potential contamination and missing controls (Perez-Muñoz et al., 2017). By definition, sterile is "devoid of life". Detecting bacteria by qPCR demonstrate the presence of bacterial DNA but not living bacteria. In the following, I will

assume that fetus *in utero* is sterile and that first colonization takes place at birth. Highly dynamic microbiota during early life will be treated separately (*cf.* Chapter I 4. Ontogeny of intestine in early life 4.1 Colonization).

In adulthood, microbiota is more stable than in early life (Borre et al., 2014; Palmer et al., 2007) and less sensitive to external insults. Indeed, highly diverse microbiota is resilient to a certain amount of time-limited stressors, as for example disease, antibiotic treatment. Resilience means that the microbiota can recover its initial state (equilibrium) after an external insult. This is due to a variety of host-microbiota and inter-community interaction at one side and on the other side host-bacteria and bacteria-bacteria antagonisms (Sommer et al., 2017). Another factor modifying microbiota is aging. Claesson et al. observed in elderly Caucasians compared to younger individuals, a shift from Firmicutes towards Bacteroidetes (Claesson et al., 2011).

1.1.2 Dysbiosis

There are several external parameters able to influence microbiota composition. For example, diet is a potent influencer of microbiota composition (Rothe and Blaut, 2013). In addition, our environment is influencing the microbial community of our gut. Medical treatment, as for example antibiotics, and disease state influence gut microbiota as well (Ottman et al., 2012). If the pressure on the microbiota ecosystem is long-lasting or more important, or if initial microbiota is less resilient, there can be a shift towards a new equilibrium, perhaps a detrimental one, also called dysbiosis (Sommer et al., 2017).

It has been shown, that microbiota is different between different geographic environments. Indeed, people in countries with westernized lifestyle and non-westernized lifestyle have distinct microbiota (Pasolli et al., 2019). Migration of Hmongs and Karens towards the United States changes the microbiota composition strongly. A loss in diversity has been observed. These changes, increasing in long-term residents and the second generation post-immigration, were associated with increased incidence of obesity (Vangay et al., 2018).

Dysbiosis is defined by Petersen and Round as a disturbance in the microbiome structure that may consist in a loss of beneficial microorganisms, and/or expansion of pathobionts or harmful microorganisms (**Figure 2**) (Petersen and Round, 2014). Gut dysbiosis is described in tremendous diseases. Autoimmune disorders (AD), metabolic disorders, inflammatory bowel diseases (IBD), irritable bowel syndrom (IBS), even psychological disorders are associated with gut microbiota dysbiosis.

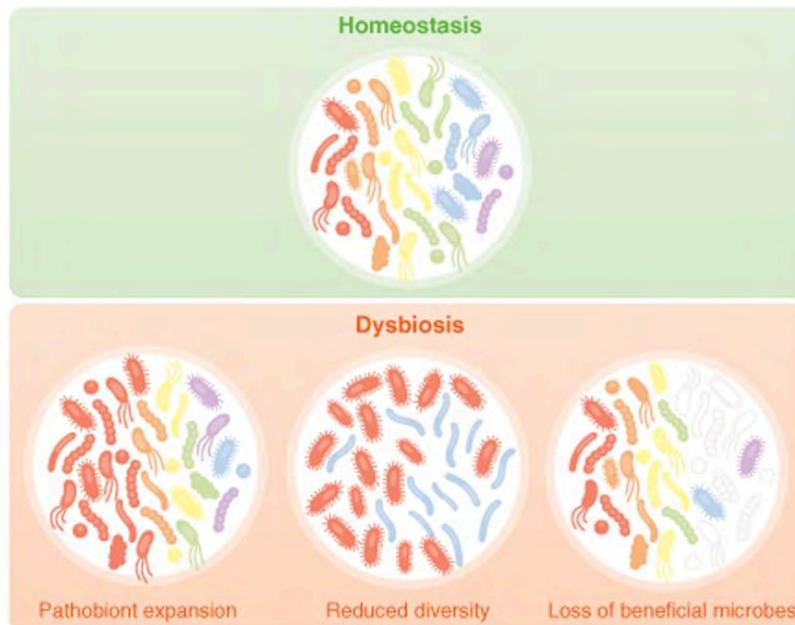


Figure 2 Dysbiosis. “A loss of beneficial microbes, expansion of pathobionts, and loss of diversity are events that encompass dysbiosis. During healthy, homeostatic conditions the microbiota is composed of a diversity organisms that are known to benefit host development and health.” (Petersen and Round, 2014)

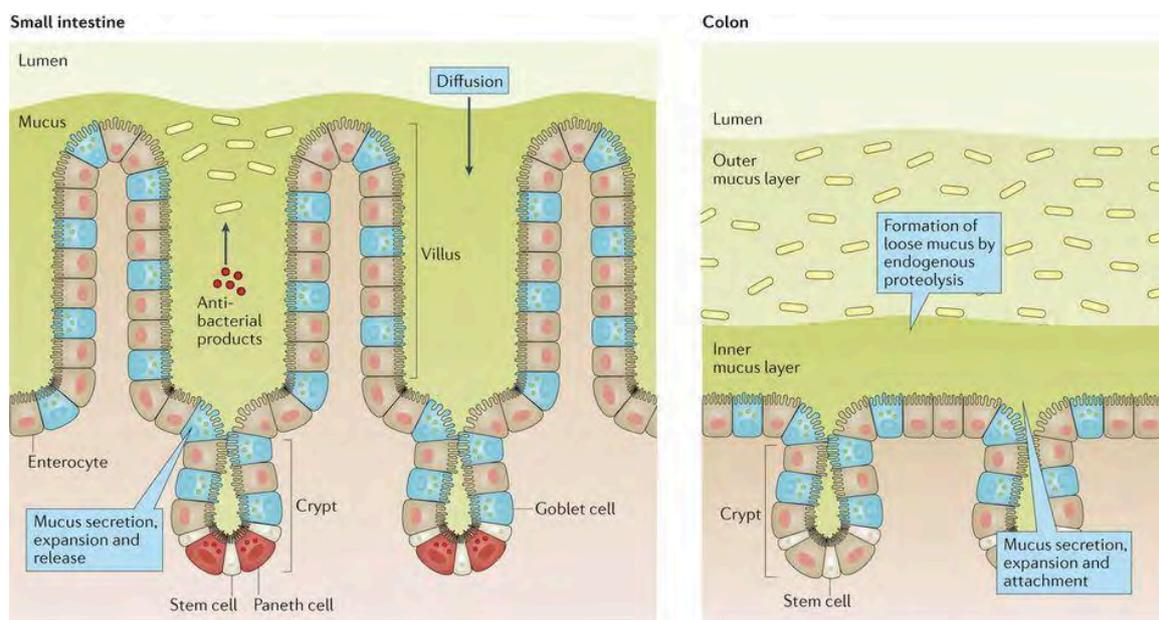
Important inter-individual microbiota composition has been observed (Franzosa et al., 2015). Further, regarding the high dynamics of microbiota during lifetime and the multiplicity of influencing factors, important questions seem to be: “Are there common elements to healthy microbiomes, in absence of overt disease? Which molecular elements of a personalized microbiome might be responsible for health outcomes, and how do they integrate with and maintain physiological processes such as the immune system and metabolism?” (the integrative HMP (iHMP) research Network consortium, 2019). The integrative Human Microbiome Project (HMP) address these question and was designed to gain a more complete view of microbial-host interactions in time. In this work frame, they found for example that not the microbiome but its prevalent molecular functions are correlated with host’s phenotype. Another finding of the more than 42 terabytes sampled omic data is that during disease microbiota is highly dynamic and could explain difficulties in cross-sectional studies to extract conclusive information (the integrative HMP (iHMP) research Network consortium, 2019).

1.2 Mucus

The gastrointestinal tract is covered by a mucus layer, protecting the epithelium from a direct contact with its microbiota. Mucus is secreted by goblet cells in the epithelial cell line and is made up mainly of mucin protein MUC2. The protein includes a high number of hydroxyl amino acids which serve as attachment sites for the O-glycans. After posttranslational modification in the Golgi apparatus, glycans represent around 80% of total mucin mass. O-

glycans can bind a great amount of water, which is giving the mucus its gel-forming property. There are also transmembrane mucins, expressed by enterocytes (MUC1, 3A/B, 4...) but in smaller quantity (McGuckin et al., 2011).

The mucus structures in small intestine and colon are different (**Figure 3**). In small intestine, there is one mucus layer; bacteria can penetrate into the mucus (Johansson and Hansson, 2016). In colon mucus is fourfold thicker and in animal models, two distinct mucus layers have been described: the outer, loose mucus layer, penetrated by gut bacteria; and the inner, firm, sterile mucus layer tightly attached to the epithelium (Atuma et al., 2001; Johansson et al., 2008).



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Figure 3 Mucus layer organization in small intestine and colon. Mucus is produced and secreted by goblet cells. The mucus layer in the small intestine is loose and not attached to the epithelium. Antibacterial products are present in mucus, limiting the penetration of bacteria towards the epithelium. In the colon, mucus is organized in outer and inner mucus layer. The outer loose mucus layer is enriched in bacteria, whereas the inner mucus layer attached to the epithelium is almost free of bacteria (Johansson and Hansson, 2016).

This vision of mucus organization, though, is debated. Notably Kamphuis et al. propose a mucus organization shaped by colonic content. They found the firm and sterile mucus layer attached to fecal pellet but not to colonic epithelium in histological staining in rat. In empty colon, the epithelium is covered with a loose, discontinuous mucus layer and devoid of bacteria (Kamphuis et al., 2017). However, Kamphuis et al. believe that the principal role of the mucus layer, even though attached to fecal pellet and not to epithelium, is the containment of bacteria in a restricted location in intestine in order to protect the host's epithelium from invasion (Kamphuis et al., 2017).

Mucus is enriched with antimicrobial peptides (Dupont et al., 2015) and secretory IgA (sIgA) (McGuckin et al., 2011), hence mucus invading bacteria will encounter antimicrobial peptides and sIgA and might be killed in this process. Indeed, it has been demonstrated that mucus has microbiota killing activity (Meyer-Hoffert et al., 2008). Invading bacteria in mucus are mainly detected by FISH technic that do not prove that bacteria are alive. The concerted action of physical, biochemical and immunological barrier components in the mucus are effective to keep bacteria away from epithelium and protect the host from infection.

1.3 Intestinal epithelium

The intestinal epithelium is formed by distinct cell types distributed along the crypt–villus axis. Although they all derived from a common stem cell progenitor located in the crypts, their morphology and roles differ (**Figure 4**) (for review (Gehart and Clevers, 2019)). The intestinal epithelium is renewed every five days, a part from Paneth cells whose lifespan has been estimated at about 60 days in mice (Ireland et al., 2005), and this constant renewing confers high plasticity and protection to the intestinal barrier since defective cells are removed rapidly (Gordon and Hermiston, 1994).

1.3.1 Different cell types in the intestinal epithelium

(a) Enterocytes

Enterocytes are present all over intestine, namely in small intestine and colon. They serve as physical barrier. Their main role is the absorption of nutrients and water. Additionally, they can also secrete antimicrobial components and they are non professional antigen presenting cells (expression of MHC2 without co-stimulatory molecules).

(b) Enteroendocrine cells

Enteroendocrine cells can be found in colon and small intestine. The high variety of enteroendocrine cells secrete different hormones. They exert regulation on intestinal motility and digestive processes but they can also influence central nervous system, via their anorexigenic effect. Some examples are mentioned in **Table 1**.

(c) Goblet cells

The mucus producing and secreting goblet cells are present all over the intestine but more abundant in colon. Goblet cells are not restricted to the production of mucus, they also can secrete antimicrobial components and play a role in antigen passage of antigens via goblet cell-associated passage (GAP), which will be developed later.

Table 1 Hormones produced by intestinal epithelial cells (examples).

Hormone	Cell	Functions (selection)
PYY	L-cells	Inhibits gastric emptying and intestinal motility; inhibits gastric acid secretion and pancreatic exocrine function; anorexigenic; stimulates mucosal enterocyte proliferation
GLP-1	L-cells	Incretin effect; delays gastric emptying; postprandial satiety
5-HT (Serotonin)	Enterochromaffin cells	Intestinal motility; intestinal secretion; visceral sensation; appetite
Somatostatin	D cells	Major inhibitory hormone for digestive endocrine and exocrine function; stimulates colonic peristalsis
GIP	K-cells	Inhibition of gastric acid secretion, stimulation of insulin secretion
Cholecystekinin (CKK)	I-cells	Anorexigenic,

(d) Paneth cells

Paneth cells are only present in the small intestine. They are located at the bottom of the crypt in proximity with intestinal stem cells. Paneth cells secrete antimicrobial peptides (AMP), as for example lysozyme. Due to their location, they also contribute to the protection of stem cells by secreting AMP highly enriched in the bottom of the crypt and thus support the stem cell niche.

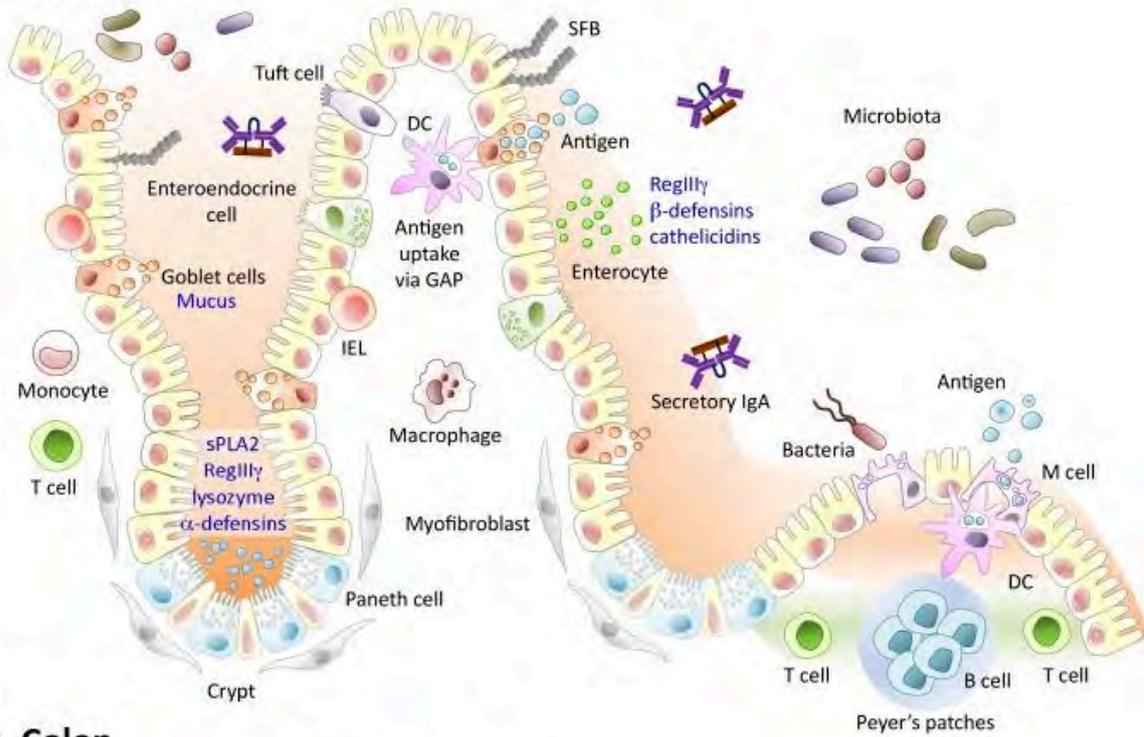
(e) M cells

Microfold (M) cells can be found in small intestine in the follicle-associated epithelium. They play an important role in the antigen uptake, facilitated by microfolds (short fold-like invaginations) on apical site. On their basolateral site, they have a large pocket promoting close contact with dendritic cells (DC) or lymphocytes. Additionally, the follicle-associated epithelium secrete less mucus, less AMP and sIgA, which help bacteria and antigens to reach M cells in order to be processed and transported for antigen presentation. Even though M cells represent a facilitating pathway for antigen to prime oral tolerance, they are not mandatory (Spahn et al., 2002).

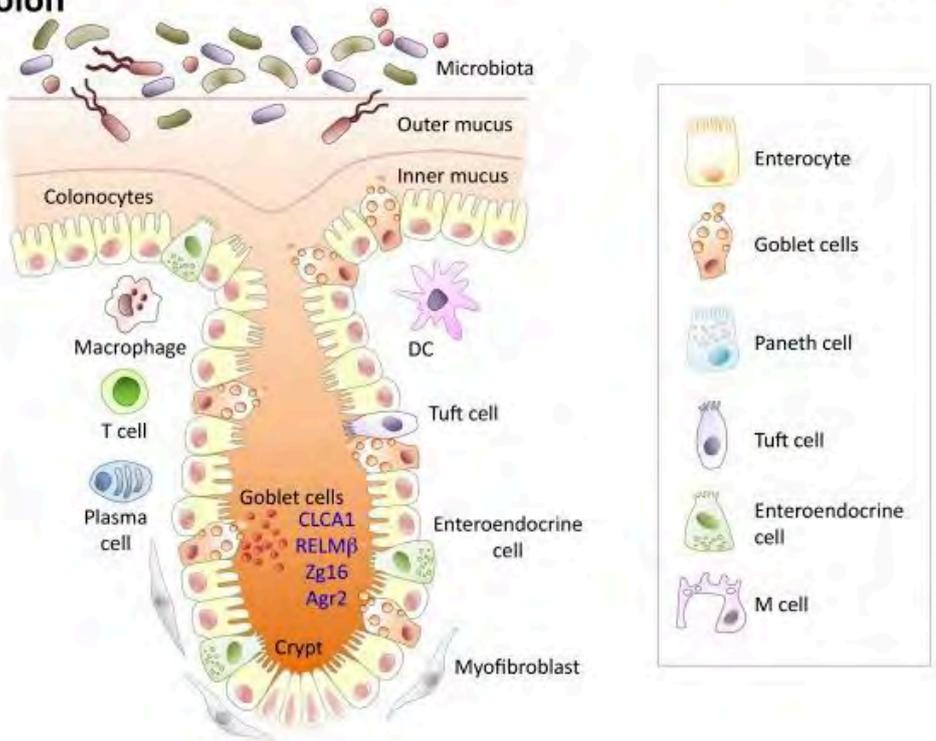
(f) Tuft cells

Tuft cells are present all along intestine. They are important for helminth detection and innate lymphoid cell 2 (ILC2) expansion (Gerbe et al., 2016). They are also responsible for opioid production and secretion in the intestinal epithelium (Gerbe et al., 2011).

(A) Small intestine



(B) Colon



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Figure 4 Different cell types in (A) small intestine and (B) colon. (A) Most of small intestinal epithelial cells are enterocytes, secreting antimicrobial peptides (RegIII γ , β -defensins and cathelicidins). Paneth cells are located at the base of the crypts, they secrete antimicrobial peptides (sPLA2, lysozyme and α -defensins). Goblet cells are secreting mucus. Antigens can enter into *lamina propria* via M cells on the surface of Peyer's Patches and via goblet cell associated passage (GAP); Underlying dendritic cells (DC) are sampling entered antigens. The *lamina propria* is rich in innate (macrophages, monocytes, dendritic cells) and adaptive (B cells, T cells, intraepithelial cells -IEL) immune cells. (B) Colonic epithelium consists mainly out of colonocytes and goblet cells. Goblet cells are forming the mucus layer via production of Muc2 but they are also secreting other proteins (RELM β , CLCA1, Zg16, Agr2). (Allaire et al., 2018)

1.3.2 Intestinal permeability

Intestinal permeability is the ability of intestinal epithelium to allow the selective entrance of luminal antigens into the organism (Ménard et al., 2010). Travis and Menzies defined intestinal permeability as “the facility with which the intestinal epithelium allows molecules to pass through by non-mediated passive diffusion” (Travis and Menzies, 2015). Intestinal permeability is expressed in cm/s. Intestinal transport is expressed in mol/h/cm² measuring a unidirectional flux. Passage of molecules from the lumen towards *lamina propria* (LP) can take place *via* paracellular diffusion or transcellular transport. Transcellular transport implicates endocytosis and exocytosis and can be receptor-mediated or not (**Figure 5**) (Ménard et al., 2010).

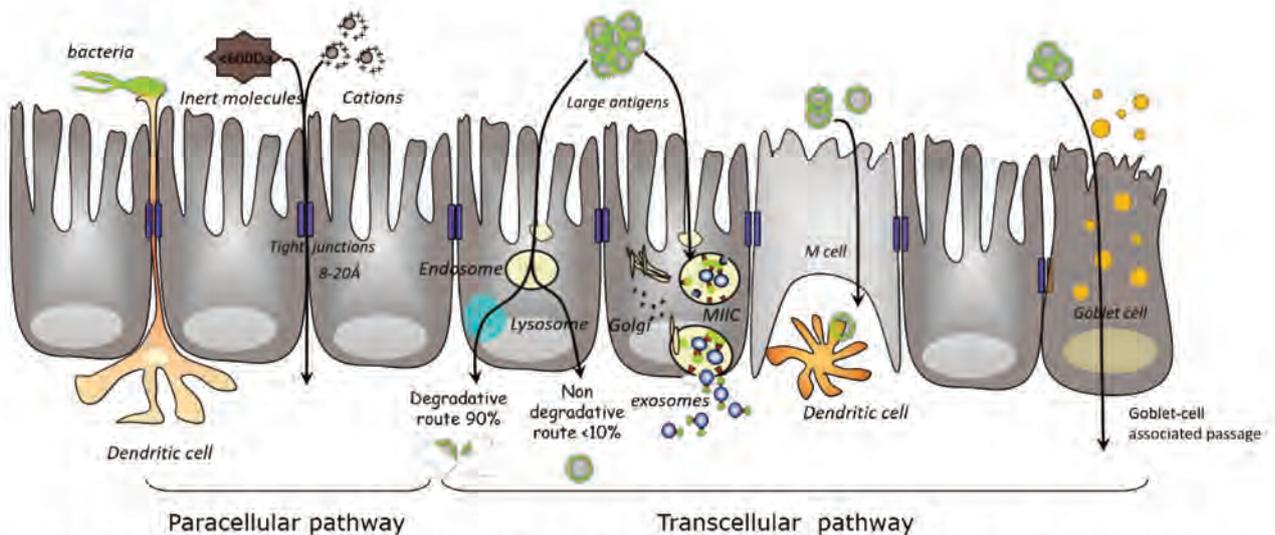


Figure 5 Pathways of intestinal permeability. From left to right: Paracellular pathways: Transepithelial antigen sensing by dendritic cells, passage of small inert molecules and cations via tight junctions. Transcellular pathways: transcellular transport of bigger molecules < 600 Da *via* the endosome and lysosome (degradative route) or not (non-degradative route), transcellular transport *via* exosomes, transcellular antigen transport *via* microfold cells (M cells) and goblet-cell associated passage (GAP). Adapted from (Ménard et al., 2010)

(a) Paracellular permeability

Paracellular passage of small hydrosoluble molecules, ions and water are regulated by tight junctions (TJ) (Turner, 2009). TJ are forming pores by which the molecules can pass by passive diffusion depending on their radius. Pore sizes are around 4-9 Å (Turner, 2009; Watson et al., 2001), limiting passage of molecules with molecular mass > 600 Da (Watson et al., 2001).

The epithelial cell line is connected by junctional proteins (tight and adherent junctions) and desmosomes, which are regulating paracellular space between adjacent epithelial cells. Tight junctions are located on lateral membrane at the apical end of intestinal epithelial cells (**Figure 6**) (Suzuki, 2013). They consist of different transmembrane protein complexes, as

claudins (Furuse et al., 1993), occludins (Furuse et al., 1998), junctional adhesion molecule (JAM) (Martin-Padura et al., 1998) and tricellulin (Ikenouchi et al., 2005). In the cell, these protein complexes are linked *via* cytosolic scaffold proteins (zonula occludens) with the perijunctional actomyosin ring. Myosin light chain (MLC) phosphorylation by MLC-kinase (MLCK) is regulating contraction and tension of actomyosin ring (Madara et al., 1987).

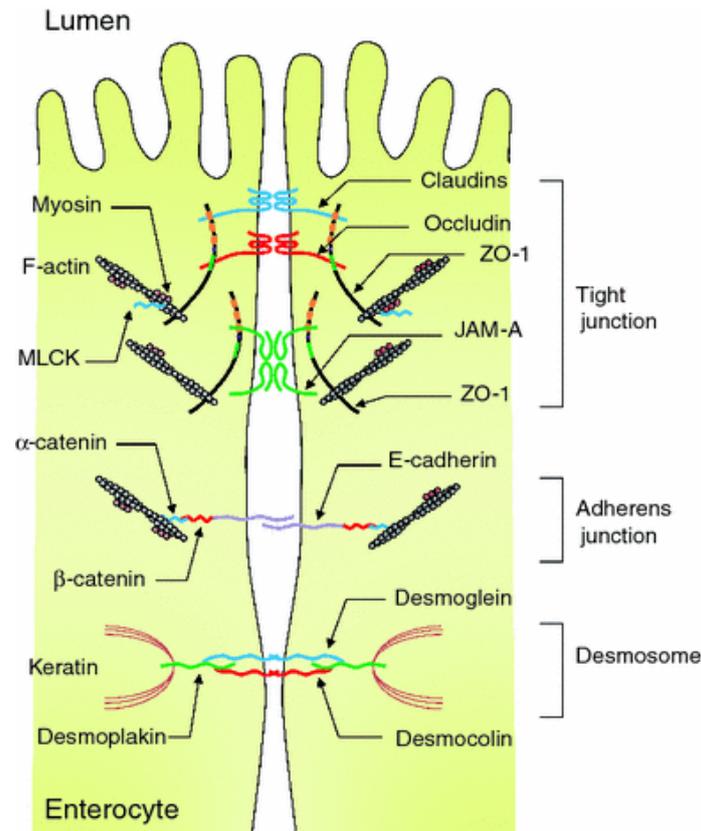


Figure 6 Intercellular junctions of intestinal epithelial cells. “The intercellular junctions are organized by different protein complexes, including tight junctions (TJs), adherens junctions (AJs), and desmosomes. TJ complexes locate at the apical ends of the lateral membranes of intestinal epithelial cells and consist of transmembrane and intracellular scaffold proteins. Extracellular loops of the transmembrane proteins (occludin, claudins, JAMs, and tricellulin) are regulating paracellular permeability. The intracellular domains of the transmembrane proteins interact with the intracellular scaffold proteins such as zonula occludens (ZO) proteins, which in turn anchor the transmembrane proteins to the actin cytoskeleton. Myosin light chain kinase (MLCK) is associated with the perijunctional actomyosin rings and regulates paracellular permeability through myosin contractility. The AJs along with desmosomes provide strong adhesive bonds between the epithelial cells and also intercellular communication” (Suzuki, 2013).

Another paracellular passage has been described by Rescigno et al. Dendritic cells (DC) can extend dendrites through the paracellular space and sample bacteria directly in the lumen. Since DC also express tight junction proteins intestinal barrier remains preserved (Rescigno et al., 2001).

(b) Transcellular permeability

Bigger molecules (> 600 Da), such as peptides and food antigens can be transported through the epithelial cell via transcellular transport route (**Figure 5**) (Gardner, 1988; Heyman

et al., 1982). Most of transcellular passage takes place in M cells, but enterocytes are also able to transport peptides. In the great majority the molecules are processed by the lysosome during their passage through cells, only a small quantity can reach LP intact (Heyman et al., 1996, 1982).

Transcellular passage can also be mediated by retro-transport of immunoglobulins from the lumen to the serosal compartment via receptor expression on the intestinal epithelium, as for example the neonatal Fc receptor (FcRn) observed in suckling rats responsible for the transmission of passive immunity by the mother (Blumberg et al., 1995; Jones and Waldmann, 1971; Simister and Mostov, 1989). FcRn has also been described on human fetal intestinal epithelial cells (Israel et al., 1997).

Goblet cells in the small intestine have been shown in the steady state, to let pass low molecular weight soluble antigens from the intestinal lumen to underlying tolerogenic-dendritic cells (DC) (McDole et al., 2012). This phenomenon is called goblet-cell associated passage (GAP). However in the colon, this antigen passage via goblet cells is restricted to a precise time window, from post-natal day 10 (PND10) to PND20 and later downregulated by microbiota in rodents (Knoop et al., 2017). As a consequence, induction of gut microbiota tolerance by the colonic immune system is crucial in early-life period and supporting the hygiene hypothesis, which proposes that the increased incidence of immune-mediated diseases is due to decreasing exposure to microorganisms in early life (Al Nabhani and Eberl, 2017; Bach, 2018).

There are multiple methods for the determination of intestinal permeability (IP) including *in vivo* and *ex vivo* measurements. First, *in vivo* methods are often used due to the simplicity of protocol and the absence for the need of complex device. Indeed, several non-metabolized molecules can be used to determine their passage across intestinal barrier *in vivo* in animal and human. The individual is receiving the molecule *via* the oral route and after a fixed time, blood or urine is collected and the marker is detected according to its properties (radioactivity, fluorescence, etc). Thanks to distinct chemical characteristics and degradation process by intestinal microbiota, different markers can be used to assess permeability of different intestinal segments (Travis and Menzies, 2015). Another possibility to assess intestinal permeability is by dosing serological markers without any previous gavage, as for example zonulin-1 (Fasano, 2011) or endotoxin (Cani et al., 2007) that will represent respectively intestinal homolog of a *Vibrio cholerae* enterotoxin that reversibly increase IP and a marker of intestinal lipopolysaccharide translocation in the blood.

1.4 Intestinal immune system

As the previous paragraph shows, the intestinal immune system is in close relationship with the other actors of the intestinal barrier and important to confer tolerance but at the same time protection to the host. Intestinal immune system is highly developed. Indeed, it is the largest immune system in the organism. There are particular structures – the gut-associated lymphoid tissue (GALT), numerous innate and adaptive immune cells and a large variety of immune active actors.

The first line of host defense is the innate immune system.

1.4.1 Antimicrobial peptides

Antimicrobial peptides (AMP) are crucial for the maintenance of intestinal homeostasis. They are controlling intestinal colonization. AMP are oligopeptides or even small proteins (5-100 amino acids), targeting a vast spectrum of microorganisms (bacteria, fungi, ..) (Bahar and Ren, 2013; Ganz and Lehrer, 1998). All AMP derive from bigger precursors and undergo important posttranslational modifications (Wilson et al., 1999). AMP repertoire is different between all species, even those who are related (Zasloff, 2002). Even different mouse strain have different AMP repertoire (Gulati et al., 2012). These differences can largely influence the microbiota and explain inter-species and inter-strain discrepancies in microbiota composition. Besides, it could confer diverse resistance capacities towards pathogens.

The presence of AMP in the intestine is also highly regionalized. AMPs are produced by enterocytes and goblet cells, but also by specialized cells, the Paneth cells. Paneth cells are only present in the small intestine, they produce and secrete α -defensins, RegIII γ , sPLA2 (phospholipase-A2), and lysozyme (Vaishnava et al., 2008).

Lysozyme can also be produced by neutrophils; it is present in saliva and maternal milk. Defensins possess three intramolecular disulfide bonds and a β -sheet structure. In vertebrates, there are two families, α -defensins, β -defensins. In mice, α -defensins are called cryptidins. Cathelin-related antimicrobial peptide (CRAMP) is member of the cathelicidin family, present in mice and similar to LL37 in human

Some AMP are targeting a fundamental differences between microbes and multicellular animals: microbes' surface is negatively charged whereas plants and animals cell membranes are composed of lipids without charge (Matsuzaki, 1999). Cationic AMP attack microbes by interaction with their membrane, lipids are displaced, membrane structure is altered as a

consequences and sometimes AMP enters into the cell. This mechanism is called Shai–Matsuzaki–Huang (Matsuzaki, 1999; Shai, 1999; Yang et al., 2000).

Due to their high aggressiveness, AMP are highly regulated and undergo numerous maturation steps. AMP are secreted either constitutively or after pattern recognition receptor (PRR) activation by microbe-associated molecular patterns (MAMPs) (Yokoi et al., 2019).

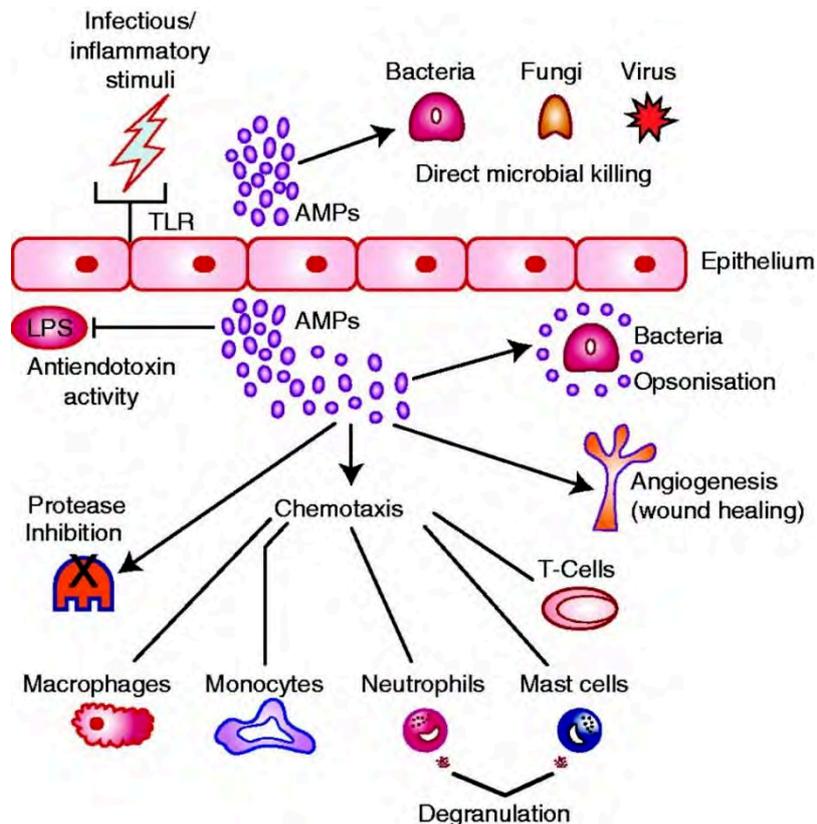


Figure 7 Functions of AMP. AMP can directly kill bacteria, virus and fungi. They can favor pathogen clearance by opsonisation of bacteria, they contribute to angiogenesis. AMP have immunomodulatory function through chemotaxis towards immune cells. They inhibit proteases and can bind endotoxin thus influencing TLR signaling (Frew and Stock, 2011).

AMP also have immunomodulatory functions (**Figure 7**). They have chemiotactic properties towards monocytes, macrophages, neutrophils and mast cells (Oppenheim et al., 2003). They can recruit T cells via induction of inflammatory cytokines (Lai and Gallo, 2009). They can favor bacterial clearance by opsonisation (Wilkinson et al., 2009). Immune response can also regulate AMP as for exemple IL-22 can induce RegIII γ release (Wang et al., 2018).

1.4.2 Innate immune cells

Additional to secreted AMP, which are regulating microbiota in the lumen, the *lamina propria* (LP) harbors a wide variety of innate immune cells, supporting the defense of host's integrity.

(a) Dendritic cells, macrophages

Dendritic cells (DCs) and macrophages are mononuclear phagocytes able to present antigens *via* major histocompatibility complex II (MHCII). In the intestine about 80% of CD11c^{hi} are CD103⁺, which is a tolerogenic phenotype of DCs, since they induce FoxP3⁺ regulatory T cell (Treg) development (Chirido et al., 2005; Coombes et al., 2007). This is underlining the fact, that tolerance to luminal antigens is of great interest throughout the intestine.

(b) Neutrophils

Neutrophils can initiate the immune response via leukotrienes and prostaglandins; kill pathogens by releasing AMPs or reactive oxygen species; or phagocyte cellular debris. They can also release IL-17 and IL-23 (Mei et al., 2012). Neutrophils also produce lipocalin-2 (lcn-2), a marker of intestinal inflammation, which is sequestering iron and thus limiting bacterial growth (Cowland and Borregaard, 1997; Yang et al., 2002) and myeloperoxidases (MPO) with great antimicrobial activity due to their oxidative potential (Serteyn et al., 2003). In the intestine neutrophils are not very numerous, unless pathological conditions, such as ulcerative colitis (Robinson et al., 1997), coeliac disease and infection (Bennouna et al., 2003).

(c) Eosinophils

Eosinophils are leucocytes with pro-inflammatory properties. Only a small percentage of circulating blood cells are eosinophils. In steady-state condition, most of them reside in the gastrointestinal tract within the LP (Powell et al., 2010; Zuo and Rothenberg, 2007). Most of them are localized in the duodenum (Mishra et al., 1999). The roles of eosinophils are multiple. Following cellular activation, eosinophils secrete toxic inflammatory mediators (proteins and peroxidase) that are stored in intracellular vesicles. These mediators destroy tissues and insert pores into membranes of target cells. By generating toxic oxygen radicals, they increase smooth muscle reactivity (Al-Haddad and Riddell, 2005). Eosinophils are also supporting plasma cell (Chu et al., 2011) function and the recruitment of DCs (Jacobsen et al., 2011). They are playing a role in inflammatory bowel disease (IBD), mainly acting as pro-inflammatory and pro-motility agent (Al-Haddad and Riddell, 2005).

(d) Mast cells

In the immune response to infection, mast cells are important effector cells. They release inflammatory mediators and recruit other immune cells, driving the pro-inflammatory immune response. Mast cells have an important role in the intestinal barrier, since epithelial function and integrity are regulated by them. Additionally, mast cells modulate innate and adaptive

mucosal immunity, and maintain neuro-immune interactions (Albert-Bayo et al., 2019). They have a great variety of receptors which permit them to react to a lot of different stimuli (chemical, microbial, neural; immune and metabolic) (Bischoff, 2007). Mast cells play a crucial role in gut-brain axis via mast cell-nerve interaction (Forsythe and Bienenstock, 2012). Additionally, they are described to play an important role in the pathogenesis of allergy. Allergen binding to serum IgE attached to mast cell's FcεRI receptors activates them, in consequence mast cells are releasing cytokines, eicosanoids and their secretory granules (Amin, 2012). In their granules they stock histamine, cytokines, granulocyte macrophage colony-stimulating factor (GM-CSF), leukotrienes, heparin, and many proteases. Those mediators are responsible for the characteristic symptoms of allergy (Amin, 2012).

(e) Innate lymphoid cells

Innate lymphoid cells (ILCs) are lymphocytes that do not express the type of diversified antigen receptors expressed on T cells and B cells. Like innate immune cells, they respond to infection quickly; but they secrete analogous inflammatory mediators as T lymphocytes. ILCs are largely tissue-resident cells, especially in mucosal surfaces, participating in tissue homeostasis. They are co-coordinating the development and maintenance of lymphoid tissue, act early during infection against pathogens and regulation of commensal bacteria. The secreted mediators of ILCs are similar to their adaptive T helper (Th) counterparts. However, ILCs are reacting to non-specific danger signals and this more rapidly than Th cells, giving them a role in the frontline of immune reaction. (for review (Eberl et al., 2015; Rankin et al., 2013)). ILCs develop in fetal liver from common lymphoid progenitors (CLPs). CLPs give rise to the lymphoid lineage: T cells, B cells and ILCs among others (Sawa et al., 2010; Vonarbourg et al., 2010). There is a great heterogeneity of ILCs; different ILCs subsets can have surface receptors and cytokine profile in common. However, they are distinct for transcription factors required and the need to classify led to three broad populations (**Figure 8**) (Spits et al., 2013; Tait Wojno and Artis, 2012).

ILC1 and natural killer (**NK**) cells respond to intracellular microbes, viruses and tumors. They produce IFN γ and are dependent on the transcriptional factor t-bet.

ILC2 are sensitive to large parasites, adipose tissue, tissue injury and allergens. They produce cytokines similar to Th2 profile, play a role in tissue repair and mucus production and are dependent on transcription factor GATA3.

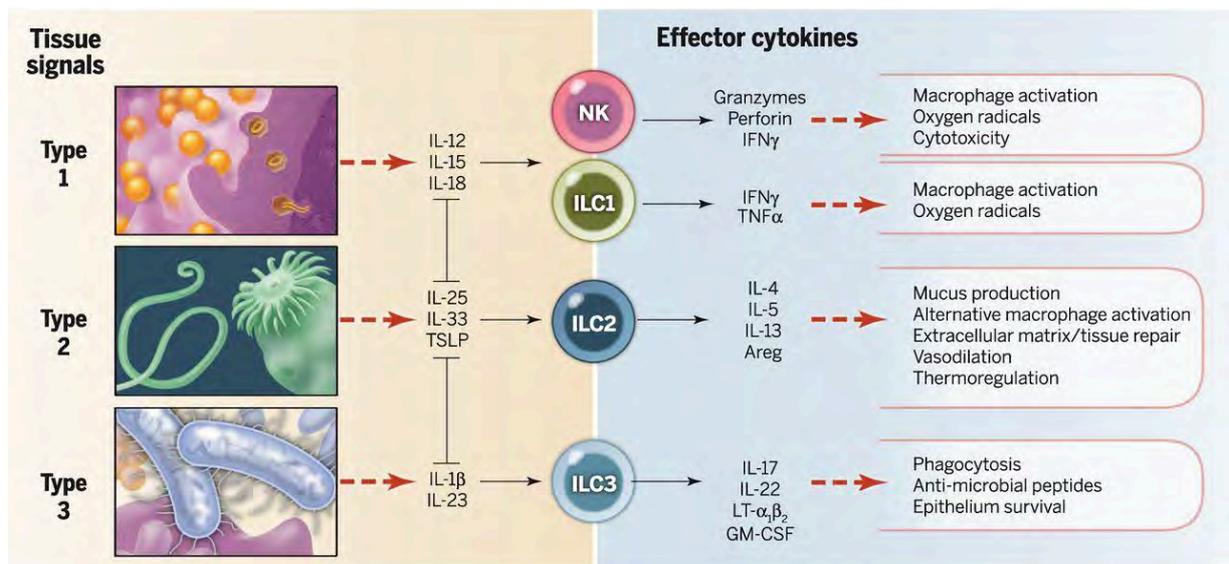


Figure 8 Innate lymphoid cells. Infected or damaged tissue are liberating signal, inducing ILC activation and expansion. Type 1 signals (intracellular microbes, viruses and tumors) induce natural killer cells (NK) and ILC1, type 2 signals (large parasites, adipose tissue, tissue injury and allergens) ILC2 and type 3 signals (extracellular microbes: bacteria, fungi) ILC3 with different effector cytokines and effector functions (Eberl et al., 2015).

ILC3 and its member lymphoid tissue inducer (**LTi**) respond to signals of extracellular microbes, bacteria and fungi. LTi are necessary for the development of secondary lymphoid tissue during fetal life. ILC3 and LTi can produce IL-17 and IL-22. ILC3 are playing an important role in intestinal homeostasis. Indeed, Lymph node resident ILC3 have been shown to present antigen to T follicular helper cells (T_{fh}), thus inhibiting the interaction of T_{fh} and B cells. As a result, mucosal IgA responses are limited. The restriction of T_{fh} response is especially developed in case of colonic antigen presentation rather than small intestine. ILC3 thus maintain tissue homeostasis and mutualism with the commensal colonic microbiota (Melo-Gonzalez et al., 2019). Another member of the ILC3 family producing IL-22 has been identified playing a crucial role in the immune response against *Citrobacter rodentium* (Sato-Takayama et al., 2008).

1.4.3 Pattern recognition receptors

Sensing of luminal exogenous fragments by the epithelium or the immune system takes place via pattern recognition receptors (PRR). Indeed, PRR, notably Toll-like receptors (TLR) and nucleotide-binding oligomerization domain (NOD) recognize and detect a variety of bacterial components (LPS, peptidoglycan, lipoproteins, flagellin, profilin, dsRNA, ssRNA and CpG DNA) (**Figure 9**). These molecules are also called microbe-associated molecular patterns (MAMPs). They are found on commensal and pathogenic bacteria. The activation of PRRs by MAMPs is initiating a cascade of signaling inside the cell, leading to gene expression of innate immune response. Consequently, a suitable immune reaction restores intestinal

homeostasis. Thus, PRRs play a crucial role in regulating the interface gut microbiota-host and in the defense of bacterial infection. They are also important for the integrity of the epithelium. Proliferation of intestinal epithelial cells has been shown to be dependent on toll-like receptor (TLR) recognition of commensal bacteria in DSS-induced colitis (Rakoff-Nahoum et al., 2004).

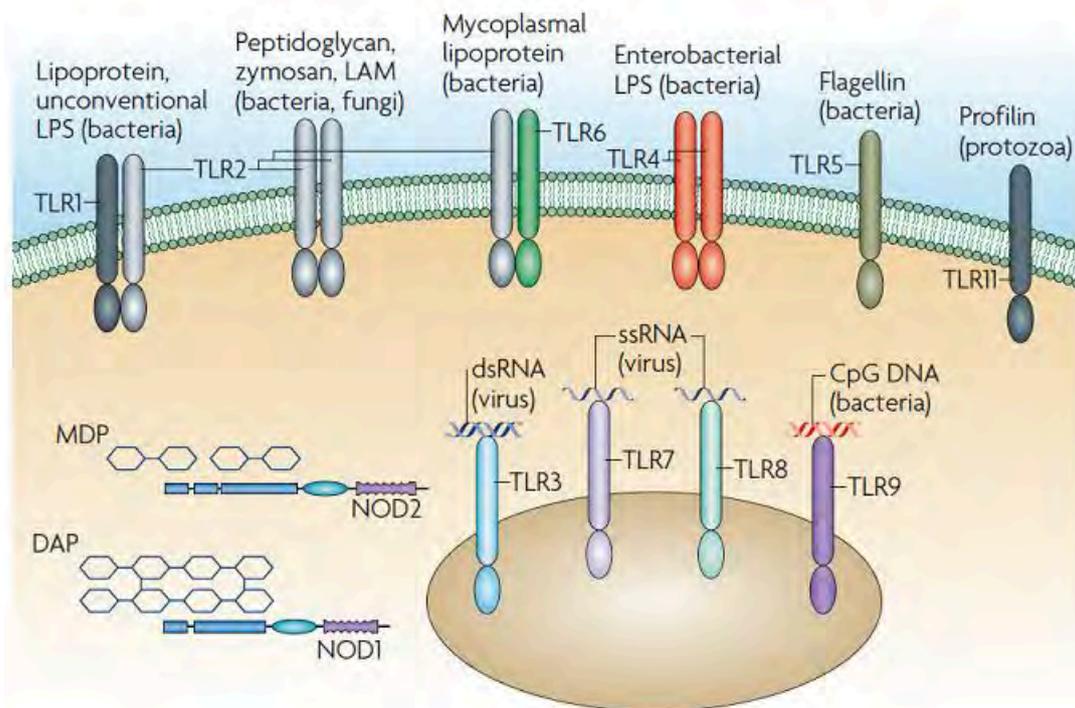


Figure 9 Pattern recognition receptors. TLR Toll-like receptors located at the cell membrane or the nucleus membrane. Intracellular NOD Nucleotide-binding oligomerization domain. DAP, diaminopimelic acid; ds, double-stranded; MDP, muramyl dipeptide; LPS, lipopolysaccharide; LAM, lipoarabinomannan; ss, single-stranded (Kaufmann, 2007).

1.4.4 GALT

The gut associated lymphoid tissue (GALT) comprises as induction sites the Peyers' patches (PPs), the appendix and isolated lymphoid follicles (ILFs) where immune cells are primed. Together with mesenteric lymph nodes (MLN) they are considered inductive sites for mucosal B and T cells. Naïve T cells from the vascularization home these structures and meet antigen-presenting cells, which arrived *via* afferent lymphatics from the inflammation sites in the gut. Naïve T cells undergo antigen-driven priming/activation, polarization, and expansion into effector cells. *Via* efferent lymphatics, they enter the lymphatic system and home to the gut *lamina propria*, the effector site, supporting the local immune response against pathogens (Figure 10, for review (Koboziev et al., 2010)).

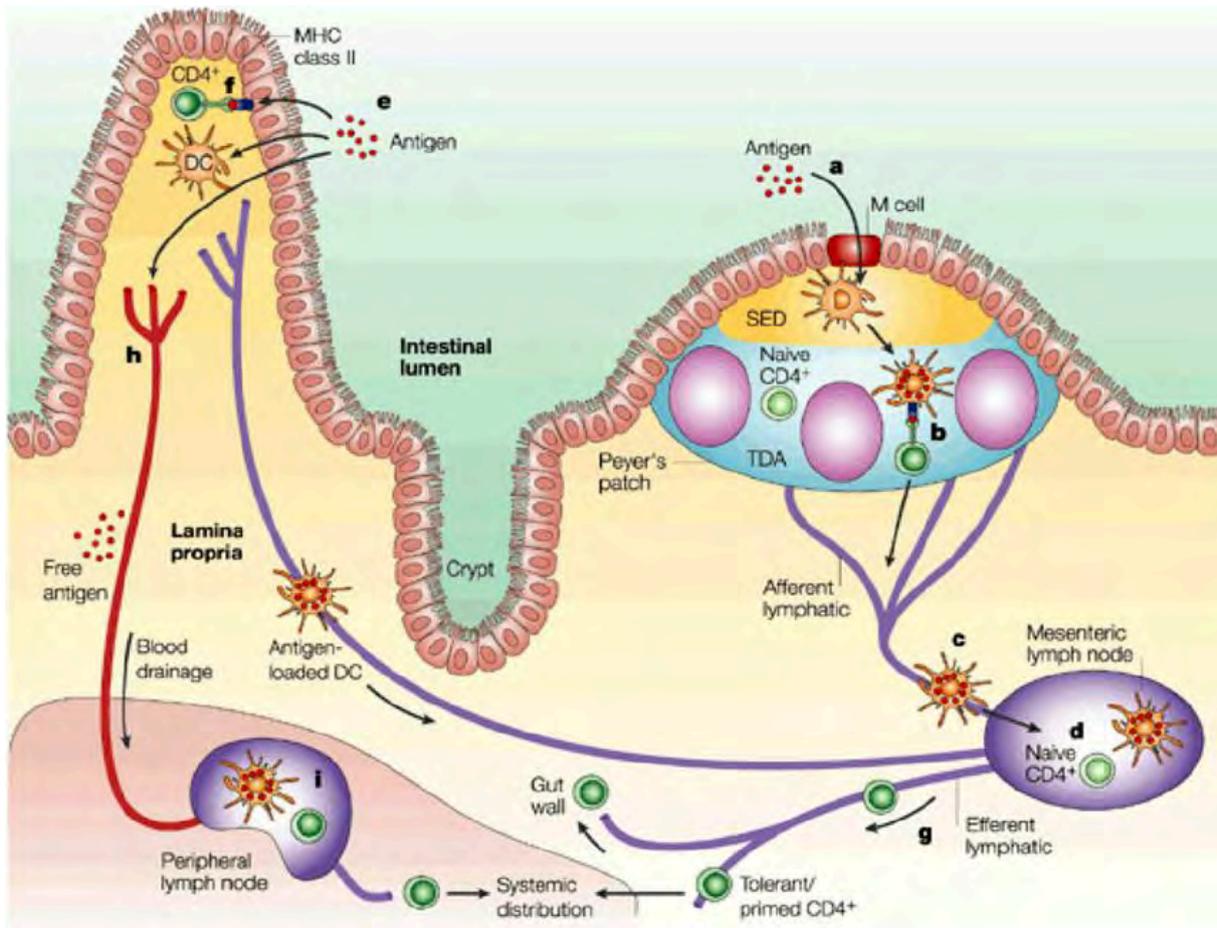


Figure 10 Inductive and effector sites of the gut-associated lymphoid tissue. (a) Antigens enter *via* M cells into Peyer's patches and are transferred to DCs, (b) and presented directly in the Peyer's patch to T cells or alternatively (c) migrate *via* the afferent lymphatics to mesenteric lymph nodes. (d) T cells will recognize presented antigens. (e) Alternatively antigens can enter directly the *lamina propria* *via* the intestinal epithelium. (f) enterocytes can serve as antigen presenting cells and directly activate T cells or antigens are transferred to DC. (g) Activated T cells enter *via* efferent lymphatics or (h) activated T cells, free antigens and DC *via* blood stream (i) peripheral lymph nodes and systemic distribution (Calder, 2013).

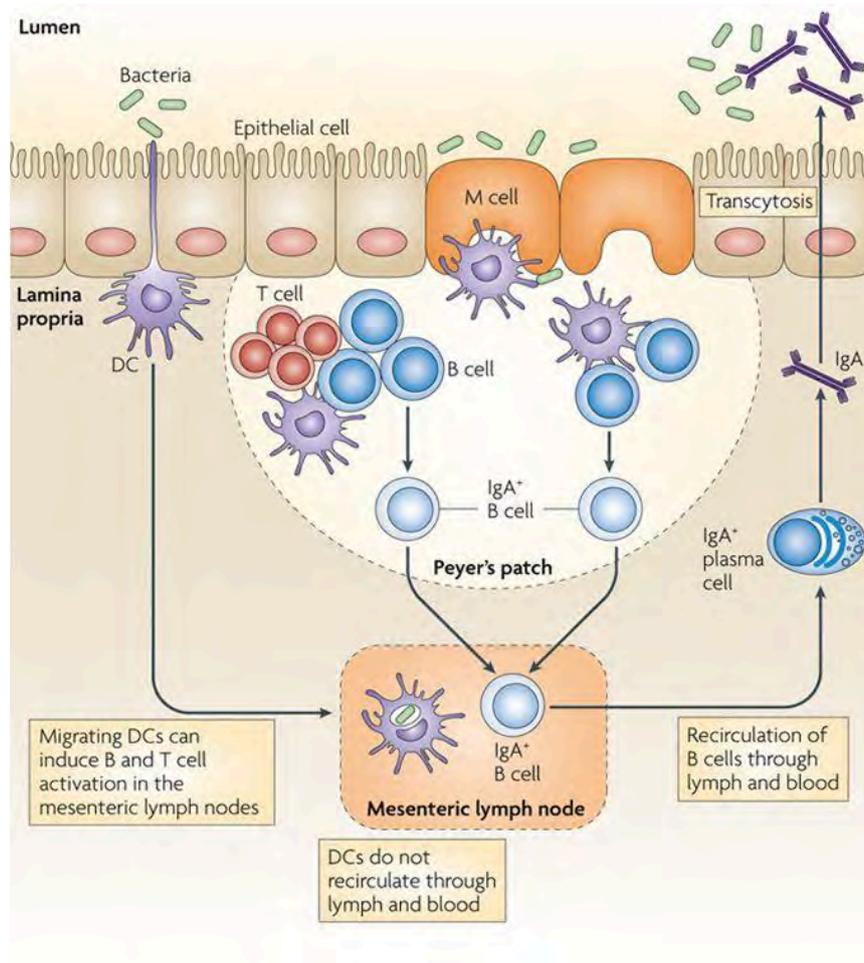
1.4.5 Adaptive immune cells

The second line of defense is the adaptive immune response. Studies in germ-free animals have shown that the adaptive immune response in the gut is extremely shaped by gut microbiota. Indeed, colonization contribute to the growth of inductive sites as PP (Shroff et al., 1995), IgA producing plasma cells expand (Benveniste et al., 1971), and the number of CD4⁺ and CD8⁺ cells increase (Guy-Grand et al., 1991). Numerous adaptive immune cells are harbored in the *lamina propria*.

(a) B cells

B cells, antibody producers, are important actors of the adaptive intestinal immune response mostly by secreting IgA. Each day 3-5 mg of antibodies are secreted into the lumen by intestinal IgA plasma cells (Brandtzaeg and Pabst, 2004). Plasma cells are most abundant in intestine, predominantly in LP of small intestine and are found in large intestine in smaller

numbers (Bowcutt et al., 2014). The humoral response in the intestine can be divided in four stages: predominant IgA induction in mucosal B cells, recirculation of IgA plasma blasts and homing into the intestinal mucosa, terminal B cell differentiation to plasma cells with local IgA production and export of IgA through the intestinal epithelial layer (**Figure 11**) (for review (Mora and von Andrian, 2008)).



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Figure 11 Cycle of B cells in the lamina propria of intestine. Antigens are sampled by DCs below M cells or through the epithelial layer by DC extensions. In Peyer's patches and mesenteric lymph nodes DCs induce B cells via antigen presentation to differentiate into IgA⁺ cells which are homing to lamina propria, secreting dimeric IgA, released into the lumen via transcytosis (Hooper and Macpherson, 2010).

The intestine is also secreting IgG, this has been shown to be exacerbated in IBD. Indeed, in intestinal mononuclear cells of IBD patients a modified Ig secretory pattern has been observed. There is a decreased spontaneous IgA secretion, but increased IgG secretion compared with intestinal mononuclear cells from healthy control (MacDermott et al., 1983, 1981). Food-specific IgE in the intestine are playing a crucial role in food allergies. Indeed, in allergic condition IgE-receptor mediated antigen transport from the lumen to the lamina propria via an IgE/allergen complex has been described to enhance epithelial antigen transport

(Bevilacqua et al., 2004; Yu et al., 2001). IgE are responsible for the activation of mast cells, triggering the allergic symptoms, as for example diarrhea (Ahmed and Fuchs, 1997).

(b) T cells

Most intestinal T cells mature in peripheral lymphoid organs. These cells gain the expression of intestinal homing receptors to migrate to the intestine. Intestinal lymphocytes are continuously exposed to food and microbial antigens. These lymphocytes help maintaining the integrity of the intestinal barrier and immune homeostasis. Due to their close location to luminal antigens they have both regulatory and effector capabilities, including the prevention of pathogenic invasion and maintenance of tolerance to prevent extensive tissue damage (for review (Hooper and Macpherson, 2010; Ma et al., 2019)).

Intestinal intraepithelial lymphocytes (**iIEL**) are in close contact with the intestinal epithelium, express the integrin CD103 (Dalton et al., 2006; Matsumoto et al., 1999) whose ligand is E-cadherin (Hadley et al., 1997) and contribute to the maintenance of barrier integrity (Culshaw et al., 1997; Inagaki-Ohara et al., 2006).

The principal CD4⁺ cells in the gut are T helper 17 (Th17), Th22 and regulatory T cells (Treg).

CD4⁺ **Th17** are producing IL-17, they appear only after colonization (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2008) and represents around 10-15% of CD4⁺ cells in SI (Maynard et al., 2007). They can stimulate mucin and AMP production, thus maintaining intestinal homeostasis.

CD4⁺ **Th22** are producing IL-22, which is promoting innate immune response (Wolk et al., 2004). In the case of allergic asthma, IL-22 has been shown to induce the expression of *REG3G* in lung epithelial cells thus improving induced asthma (Ito et al., 2017). Also in murine intestinal epithelial cells, IL-22 induced the expression of the AMP *REG3G*. Additionally, IL-22 increased paracellular permeability in Caco-2 cell culture (Wang et al., 2017). Decreased Th22 cells are described in *lamina propria* mononuclear cells of patients suffering from ulcerative colitis compared to healthy control (Leung et al., 2014).

CD4⁺ **Th1** produce IFN γ , which is enhancing macrophage activity.

Regulatory T cells (**Treg**) are crucial to regulate containment of immune response. Treg are under the control of transcription factor FoxP3⁺ and produce the cytokines IL-10 and TGF β .

CD4⁺ Th2 have a role in allergy, tissue repair and parasite infection. Th2 are crucial for the fight against helminth infection. Th2 stimulate intestinal functions in order to kill and expulse the multicellular parasite by inducing smooth muscle hypercontractility, enhanced mucus secretion and activation of intestinal mast cells (Bamias and Cominelli, 2015). However, over-activation of Th2 response can lead to excessive collagen deposition, probably responsible for fibrogenesis in ulcerative colitis and Crohn's disease (Principi et al., 2013).

1.5 Intestinal motility

As seen in the previous paragraph, intestinal motility is an important actor of intestinal barrier, since it is crucial for the expulsion of pathogens or toxic substances. There are two smooth muscles layers enveloping the intestine, the inner, circular muscle and the outer, longitudinal muscle (**Figure 12**). The main task of intestinal muscles is the aboral transport of the bolus from esophagus to anal sphincter by longitudinal muscle contraction above and relaxation below bolus (peristalsis). On the other side, contractions and segmentations by circular muscles favor also the mixing of the luminal content with the digestive juice. (Kumral and Zfass, 2018)

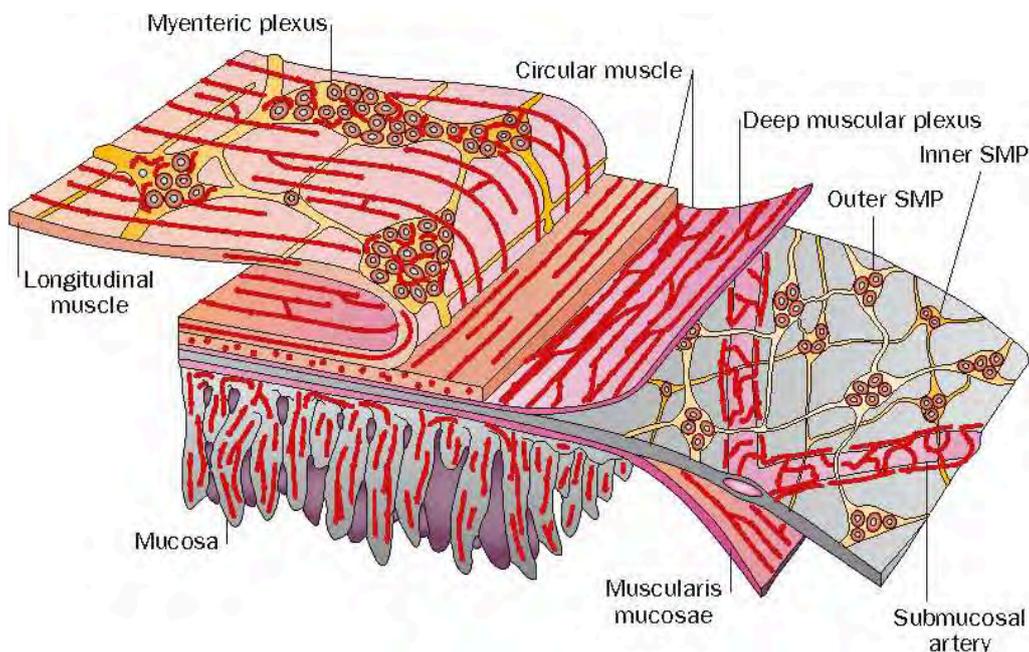


Figure 12 The enteric nervous system. The ENS consists of the myenteric plexus, located between the longitudinal and circular muscle, and the submucosal plexus (SMP, Meissner plexus) located between the *muscularis mucosae* and the circular muscle (Furness, 2012).

The intestinal motility is autonomously regulated by the enteric nervous system (ENS). The movements are initiated by distention of the intestinal tube and triggered by the myenteric plexus, located in between both muscle layers. The sympathetic and parasympathetic nervous

system, elements from the autonomous nervous system (ANS), have only modulating influence on gut motility.

2. Enteric nervous system

The enteric nervous system (ENS) is located in the wall of digestive tube (**Figure 12**). The ENS in human contains 200-600 million neurons, which are distributed in many thousands of small ganglia. They can be found in the myenteric and submucosal plexuses. The myenteric plexus is located between the two smooth muscle layers, covering the intestine from esophagus to anal sphincter. The efferent limbs end in the smooth muscle layer, stimulating peristalsis and segmentation. Submucosal ganglia are present in the small and large intestine. It is regulating secretory functions of epithelial cells in the mucosa (Furness et al., 2014). There is close communication between the ENS and the central nervous system (CNS) and the ANS and also regulation by numerous hormones.

3. Gut-Brain Axis

“The gut-brain axis consists of bidirectional communication between the central and the enteric nervous system (ENS), linking emotional and cognitive centers of the brain with peripheral intestinal functions” (Carabotti et al., 2015). The gut-brain axis includes the central nervous system (CNS), the autonomic nervous system (ANS) including sympathetic and parasympathetic limb, ENS and the hypothalamic pituitary adrenal (HPA) axis (**Figure 13**). The HPA axis is playing a crucial role in the response to stressors (*cf.* Chapter II Stress).

Top-down communication appear through neurons (vagus nerve) and hormones (cortisol) which can influence gut motility, immunity, intestinal permeability and mucus production. Bottom-up communication happens via hormones (5-HT), cytokines produced by immune cells and mast-cell neuron interaction. In case of invading microorganisms in the periphery, pro-inflammatory cytokines are produced by activated neutrophils and macrophages and shown to induce in brain sickness behavior (Konsman et al., 2002). These findings underline the role of cytokines in communication with CNS. Microbiota can also influence gut-brain axis *via* metabolites as for example SCFAs and microbiota-produced or degraded neurotransmitter. Indeed, bacteria can produce themselves a big variety of neurotransmitter (Lyte, 1993) but also influence host's neurotransmitter. Serotonin (5-HT) is decreased in GF colon and blood (Wikoff et al., 2009) and can be restored by colonization. Spore-forming bacteria have been shown to stimulate 5-HT secretion by enterochromaffin cells via different SCFAs, among them butyrate and propionate (Yano et al., 2015). 5-HT plays a crucial role in gut motility, thus a restoration of 5-HT levels by gut microbiota modifications could be

beneficial in gastro-intestinal disorders associated with motility. Indeed, Yano et al. showed that colonization with their spore-forming bacteria increases gut motility in GF mice (Yano et al., 2015).

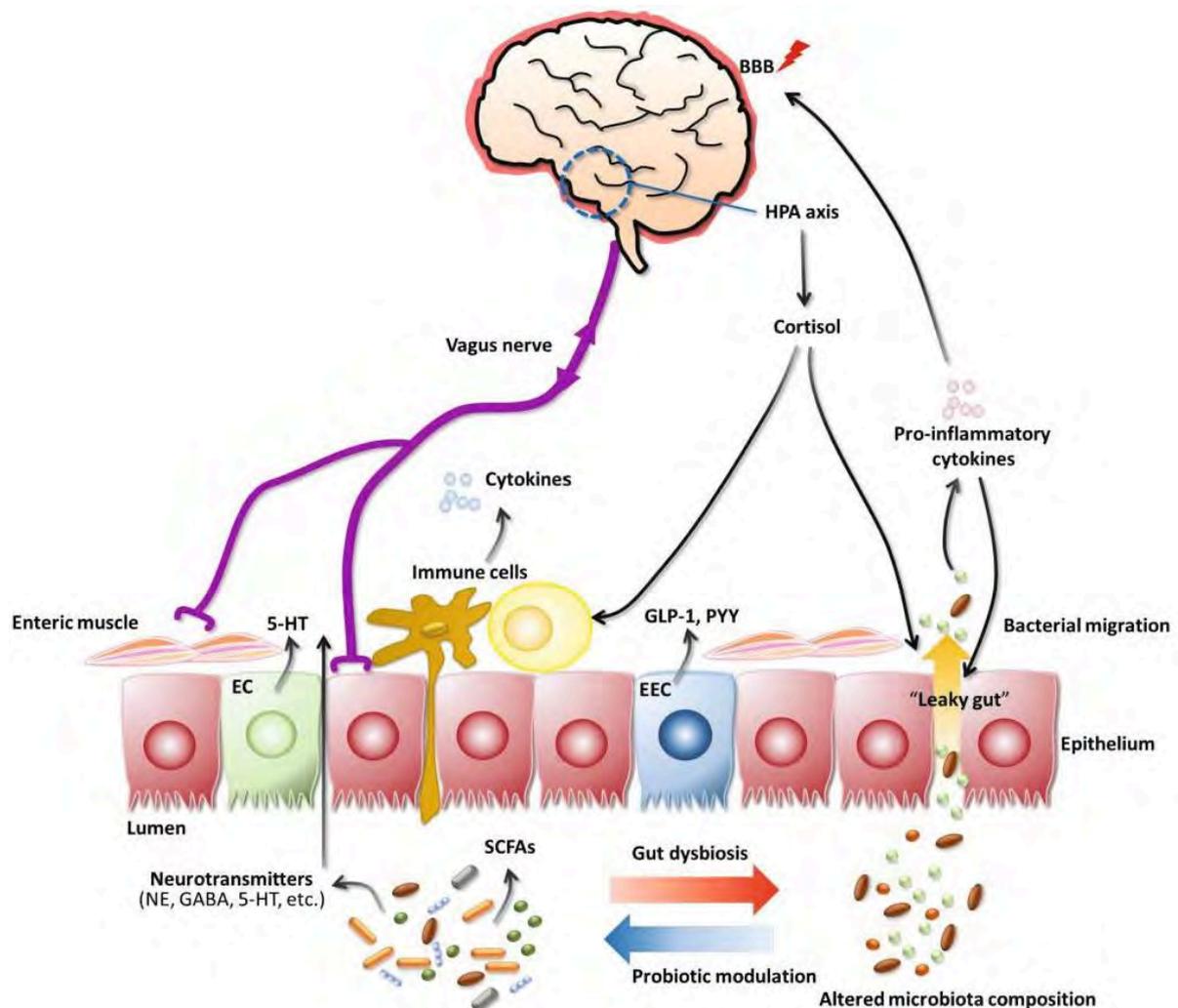


Figure 13 The gut-brain axis. Top-down communications *via* 1) vagus nerve which is enervating enteric muscles and sending signals to intestinal epithelial cells, 2) Hypothalamus-Pituitary-Adrenal axis by cortisol, which is influencing immune cells, intestinal permeability. Bottom-up communication by 1) intestinal hormone secretions (5-HT by EC and GLP-1 and PYY by EEC), 2) cytokine secretion by immune cells. Microbiota is also playing a role in gut-brain axis by the production of SCFAs and neurotransmitters, which can influence intestinal epithelium but also enter into the system. NE, norepinephrine; GABA, γ -aminobutyric acid; BBB, blood brain barrier; EEC, enteroendocrine cell; EC, enterochromaffin cell; GLP-1, glucagon-like peptide-1; PYY, peptide tyrosine tyrosine; 5-HT, 5-hydroxytryptamine; SCFAs, short-chain fatty acids (Kim et al., 2018).

4. Ontogeny of intestine in early life

At birth, the intestine is immature, especially in rodents, and needs to undergo various maturation processes. The organism has to face colonization by microorganism, must develop immune tolerance to commensal and food antigens and establish a robust immune response against pathogens.

4.1 Colonization

A first great change in the neonatal gut is the colonization by microbiota. *In utero*, the organism is sterile and at birth, it is facing microorganism for the first time. Delivery mode is greatly shaping microbiota in the newborn. Infants born by vaginal delivery are colonized by vaginal microbiota of the mother, whereas infants born by caesarian section (CS) have first gut microbiota similar to skin microbiota of the mother (Dominguez-Bello et al., 2010). During the first days microbiota is evolving, at 1 week of age, microbiota of neonates vaginally delivered is characterized by abundant levels of *Bifidobacterium* and *Bacteroides*. Infants delivered by CS have a microbiota characterized by blooming *Clostridium* (Hesla et al., 2014).

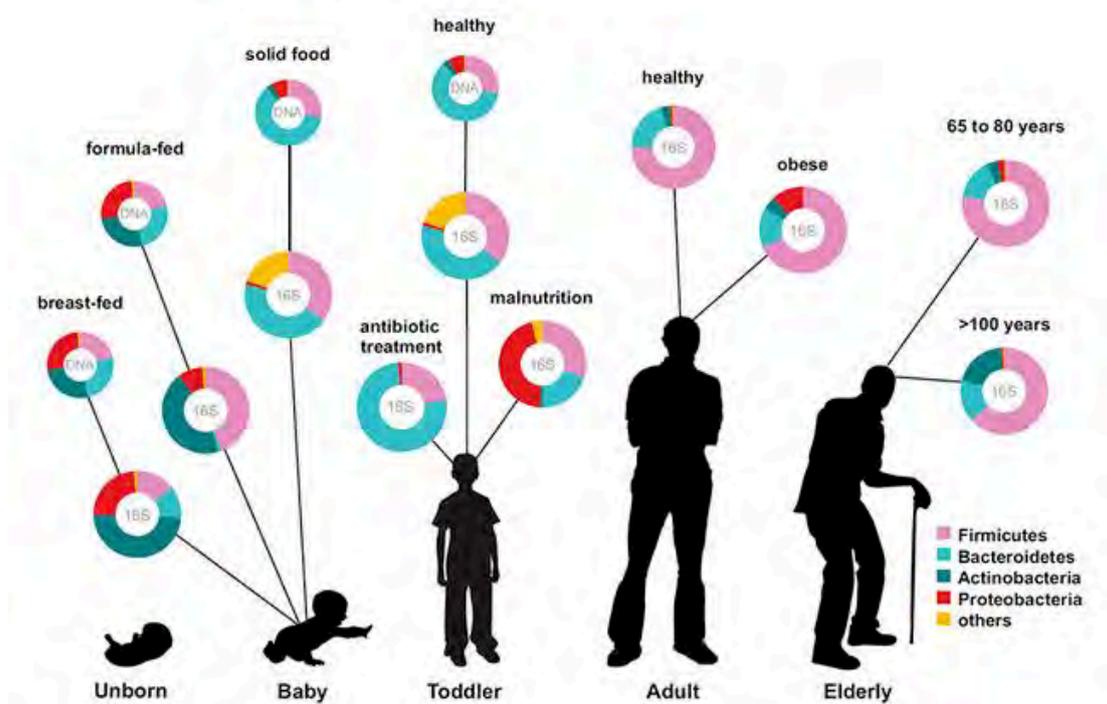


Figure 14 Intestinal microbiota composition over lifetime. “The graph provides a global overview of the relative abundance of key phyla of the human microbiota composition in different stages of life. Measured by either 16S RNA or metagenomic approaches (DNA)” (Ottman et al., 2012).

Early life nutrition affects also microbiota composition. Breast-fed and formula-fed children have distinct microbiota. Introduction of solid food induces important changes in microbiota composition (Hill et al., 2017). *Bifidobacteria* diminish with introduction of solid food and microbiota is more and more similar to classical adult-like microbiota (Palmer et al., 2007). Aerobic and facultative anaerobic bacteria will decrease in favor of anaerobic species, the postulate is, that facultative anaerobic bacteria prepare the intestinal niche for strict anaerobic bacteria by diminishment of intestinal oxygen (Jost et al., 2012; Timmerman et al., 2017). At adulthood, anaerobic bacteria are dominant in the gut (**Figure 14**).

4.2 Gut closure

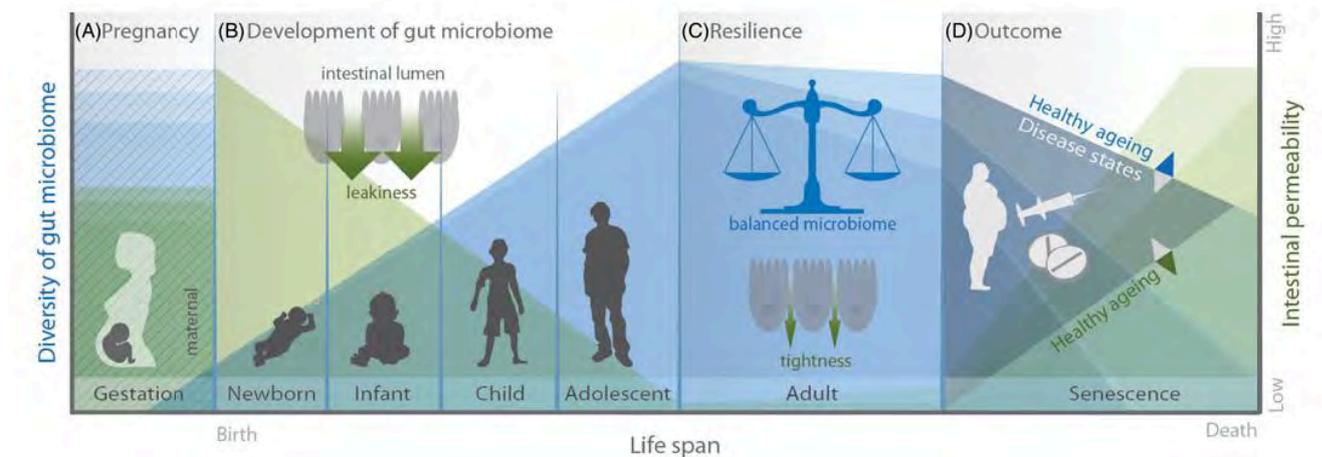


Figure 15 Intestinal permeability and gut microbiota diversity in lifetime. “Starting early in life, environmental factors influence microbial diversity (blue) and gut permeability (green), affecting the duration of resilience and metabolic health (different shadings). (A) During pregnancy, the developing foetal gut is ‘primed’ by the maternal gut microflora and intestinal permeability (diagonal lines), particularly towards the latter stages of gestation and prior to the imminent microbial colonization post-birth. (B) Following birth, the gut is colonised with bacteria and diversity develops throughout infancy and childhood with a subsequent decrease of the initially very high intestinal permeability. (C) In adulthood, events in early life have combined with tight intestinal permeability to influence the ‘resilience’ of adult gut’s microflora. This is positively correlated with diversity and determines the health trajectory for the remaining lifespan. (D) In later years, after a period of ‘resilience’, the diversity in the gut microbiome declines and the leakiness increases during normal ageing. A low diversity or high leakiness can lead to the accelerated decline and places the individual at risk of developing a range of chronic metabolic diseases” (Kerr et al., 2015).

During the first days of our life, the gut is hyperpermeable in order to receive maternal nutritional compounds but also immune modulatory mediators (van Elburg et al., 2003). In rats, intestinal permeability measured in vivo by FITC-Dextran 4 kDa gavage, has been shown to decrease constantly from 10 days after birth to 50 days after birth (Moussaoui et al., 2014). Intestinal hyperpermeability in neonates is a normal function (Catassi et al., 1995; Weaver et al., 1984) However, in premature infants intestinal permeability is excessively increased (Weaver et al., 1984), which can lead to uptake of pathogens and infection in pathogenic context (Insoft et al., 1996). The physiological decrease in intestinal permeability after birth is called “gut closure” (**Figure 15**) (Drozdowski et al., 2010). The work of Vukavic et al. suggest that early beginning of breastfeeding (1h-6h after birth) leads to a more rapid gut closure than late start of breastfeeding (12h-15h after birth) (Vukavić, 1984), underlining the impact of breastmilk on intestinal permeability. The start time of breastfeeding influences the composition of the microbiota through maternal Ig and AMP present in high concentrations in colostrum (Menchetti et al., 2016).

4.3 Development of intestinal barrier

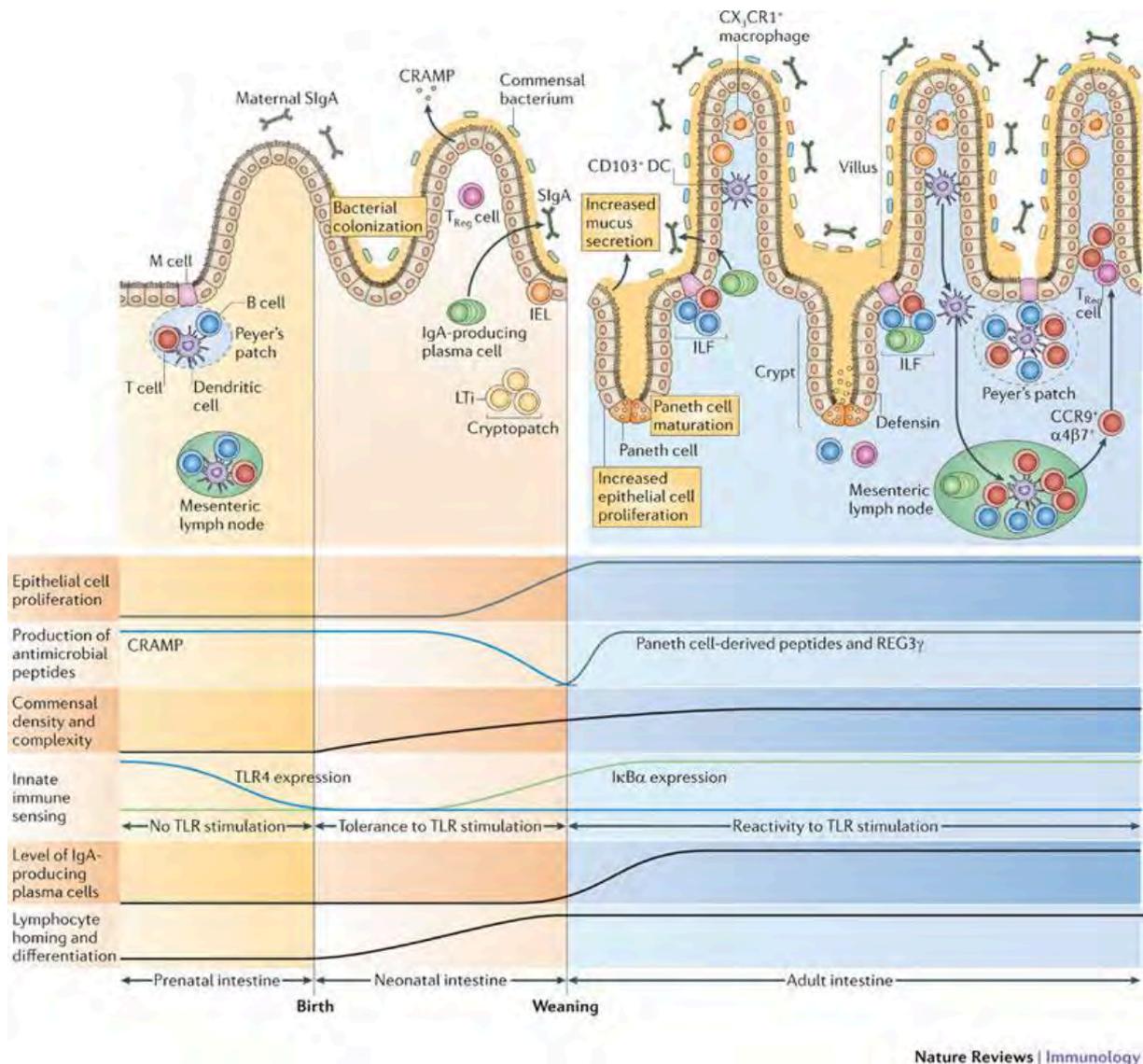


Figure 16 Ontogeny of intestinal barrier in rodents. The intestinal barrier is highly dynamic in early life. Immature prenatal intestine matures after birth and with weaning towards adult intestine. Epithelial cells proliferation increases around weaning, crypts appear. Prenatal and neonatal CRAMP production ceases and is replaced by Paneth cell derived peptides. Paneth cells appear with weaning. Bacterial colonization takes place from birth on and microbiota gains in diversity and density. Innate immune sensing undergoes maturing processes as well. TLR4 and I κ B α expression evolve during early life. IgA-producing plasma cells appear and replace secretory IgA delivered through breastmilk by the mother. Lymphocyte homing and differentiation takes place from birth on (Renz et al., 2011).

The expression of AMP undergoes a switch in the first days of life. In rodents, during the first days the AMP CRAMP is constitutively expressed in neonates gut epithelium and regulates the microbiota. The intestine mature, microbiota is diversifying. Crypts that are absent in neonatal rodent intestinal epithelium appear with time (Hirano and Kataoka, 1986), CRAMP disappears after the first 15 days and is replaced by Paneth cells with larger AMP repertoire. Paneth cells are localized in the bottom of the crypts and appear only 15 days after birth (Ménard et al., 2008) coinciding with microbiota expansion and diversification of food. In this period,

intestinal epithelial cell proliferation accelerates (van der Flier and Clevers, 2009), and crypts multiply. Paneth cells are playing an important role in the fision of one crypt into two daughter crypts (Langlands et al., 2016). All this changes in intestinal physiology coincide with the changes around the weaning period: introduction of solid food and diversification of diet and consequently arrival of new commensal bacteria in the gut (**Figure 16**).

In human, intestine is more mature at birth. Crypts are formed; Paneth cells and Peyer's patches are present. However, the intestinal immune system, structurally complete, undergoes great maturation and expansion during this period (Stockinger et al., 2011). The neonatal immune response is distinct from adult one. Indeed, CD4⁺ cells tend to differentiate into regulatory T cells in response to stimulation (Wang et al., 2010). B cells expand in plasma cells, producing sIgA, important for the neutralization of nutritional and microbial antigens (Pabst et al., 2008).

4.4 Glucocorticoide sensitivity

Moussaoui et al. described that glucocorticoid receptor (GR) expression in colon of rat pups (PND10) is significantly higher than in PND20 rats. This increased expression comes along with higher sensitivity to dexamethasone a GR agonist in PND10 vs PND20 pups. The higher sensitivity for glucocorticoids leads to increased intestinal permeability in PND10 but not PND20 pups in response to a single maternal separation stress (Moussaoui et al., 2014). Intriguingly, dexamethasone or hydrocortisone are often used to prevent bronchopulmonary dysplasia in preterm children. Effects of these GR agonists on gut has to be assessed. Indeed, preterm gut resembles PND10 rat pups gut in maturity and glucocorticoid sensitivity. Morris et al. report in a meta-analysis that early hydrocortisone treatment in preterm increases significantly the risk for intestinal perforation (Morris et al., 2019). Thus, clinical practice should be probably reconsidered in order to develop save long-term treatment for neonates, taking into account their special physiology.

CHAPTER II

GLUCOSE METABOLISM

In the previous chapter, I presented the different components of the intestinal barrier and their development in early life period. During my PhD, we were also interested in the effects of early life stress on glucose metabolism. First, we were focused on metabolic disorders characterized by glucose intolerance associated with insulin resistance and obesity. Afterwards, we were interested in autoimmune diabetes.

1. Regulation of blood glucose

The regulation of blood glucose levels is crucial for survival and for that reason submitted under tight control. Various hormones and neuropeptides are implicated in the regulation of glycaemia. They are released mainly from the brain, pancreas, liver, and intestine as well as adipose and muscle tissues.

1.1 Regulation by pancreatic hormones

Pancreas is a key player in the regulation of blood glucose levels, secreting insulin and glucagon. Insulin, at one hand, has a blood-glucose lowering effect, glucagon, at the other hand, a glucose mobilizing glucose from tissue towards the circulation. Both hormones are produced in islets of Langerhans in the pancreas, insulin from pancreatic β -cells, glucagon from α -cells.

In postprandial state, insulin is released in response to glucose entry via GLUT2 into the β -cell. ATP/ADP ratio is increasing, ATP-sensitive K^+ channel close and as a consequence membrane is depolarized, voltage-dependent Ca^{2+} -channel open and increasing Ca^{2+} levels lead to fusion of vesicles of insulin with the membrane (for review (Röder et al., 2016)). Insulin is stimulating glucose uptake by peripheral organs (liver, muscles, adipose tissue) via insulin receptor whose activation stimulates GLUT4 translocation to the membrane. In liver, insulin stimulates glycogenesis and inhibits gluconeogenesis (**Figure 17**).

In fasted state, glucose levels are decreasing and glucagon is secreted, which stimulates gluconeogenesis and glycogenolysis in liver in order to liberate glucose to normalize blood glucose levels (**Figure 17**).

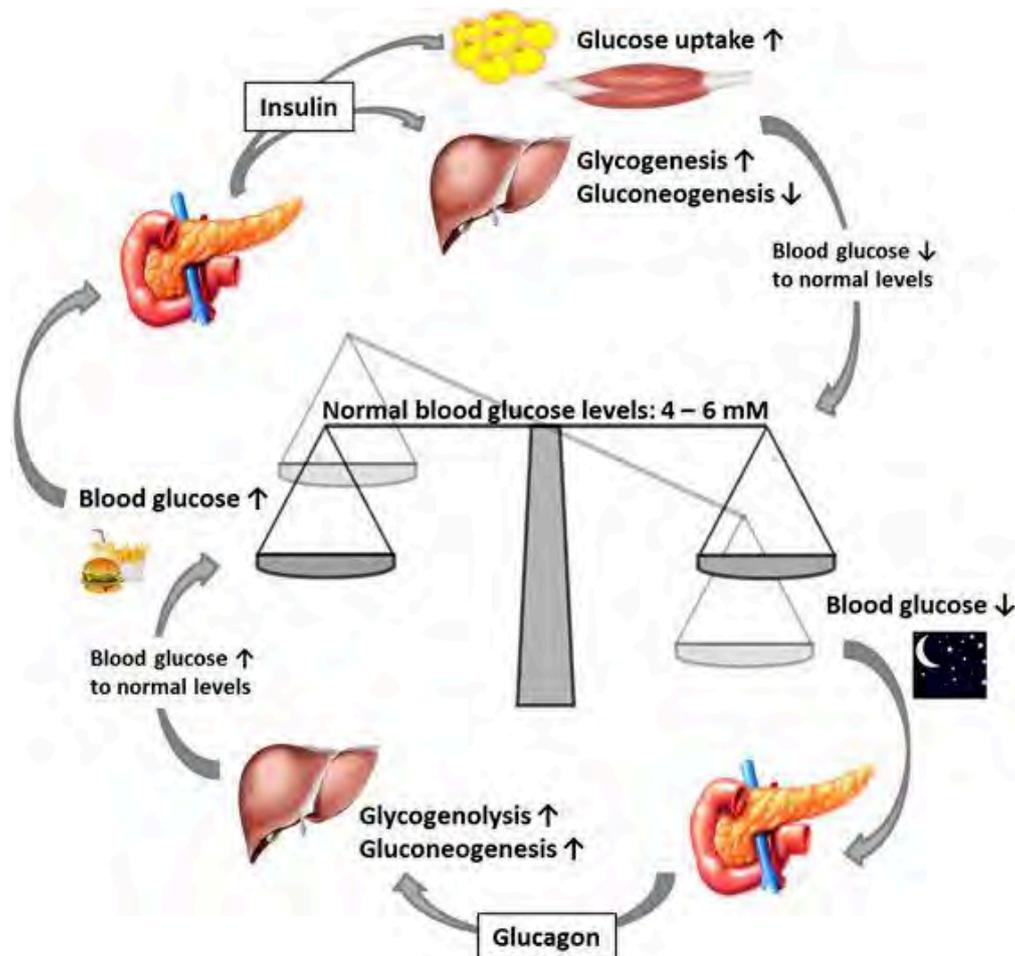


Figure 17 Glucose metabolism. After a meal blood glucose is rising, GLUT2 receptors in pancreatic β -cells trigger insulin release, which stimulates glucose uptake in peripheral organs (adipose tissue, muscles). In liver insulin upregulates glycogenesis and downregulates gluconeogenesis. These processes lead to normalization of blood glucose levels. During night, when blood glucose diminishes, pancreatic α -cells release glucagon. Glucagon stimulates glycogenolysis and gluconeogenesis in the liver and as a consequence restores normal blood glucose levels (Röder et al., 2016).

1.2 Role of gut hormones in blood glucose regulation

Besides pancreatic regulation of glucose metabolism there are numerous intestinal hormones playing a role in glucose homeostasis. **Ghrelin** is secreted by stomach mucosa in fasted state (Ariyasu et al., 2001). It can also be secreted by endocrine pancreas. Ghrelin plays an orexigenic role (Nakazato et al., 2001) but influences also gastric emptying, growth hormone release and body weight regulation (Verhulst and Depoortere, 2012). Ghrelin has an inhibitory function on insulin secretion and therefore a role in glucose metabolism (Broglio et al., 2001; Dezaki et al., 2008). **GIP** (glucose-dependent insulinotropic polypeptide) and **GLP-1** (glucagon-like peptide-1) are important incretins regulating blood glucose. GIP is secreted by K cells and GLP-1 by L cells. Both have important roles in the regulation of postprandial blood glucose levels. Indeed, they stimulate insulin secretion and contribute to glucose homeostasis

(for review (Nauck and Meier, 2018)). In diabetic state, a reduced incretin effect, characterized by GLP-1 and GIP resistance, has been observed (Nauck et al., 1986; T. et al., 2002).

2. Metabolic syndrome and associated complications

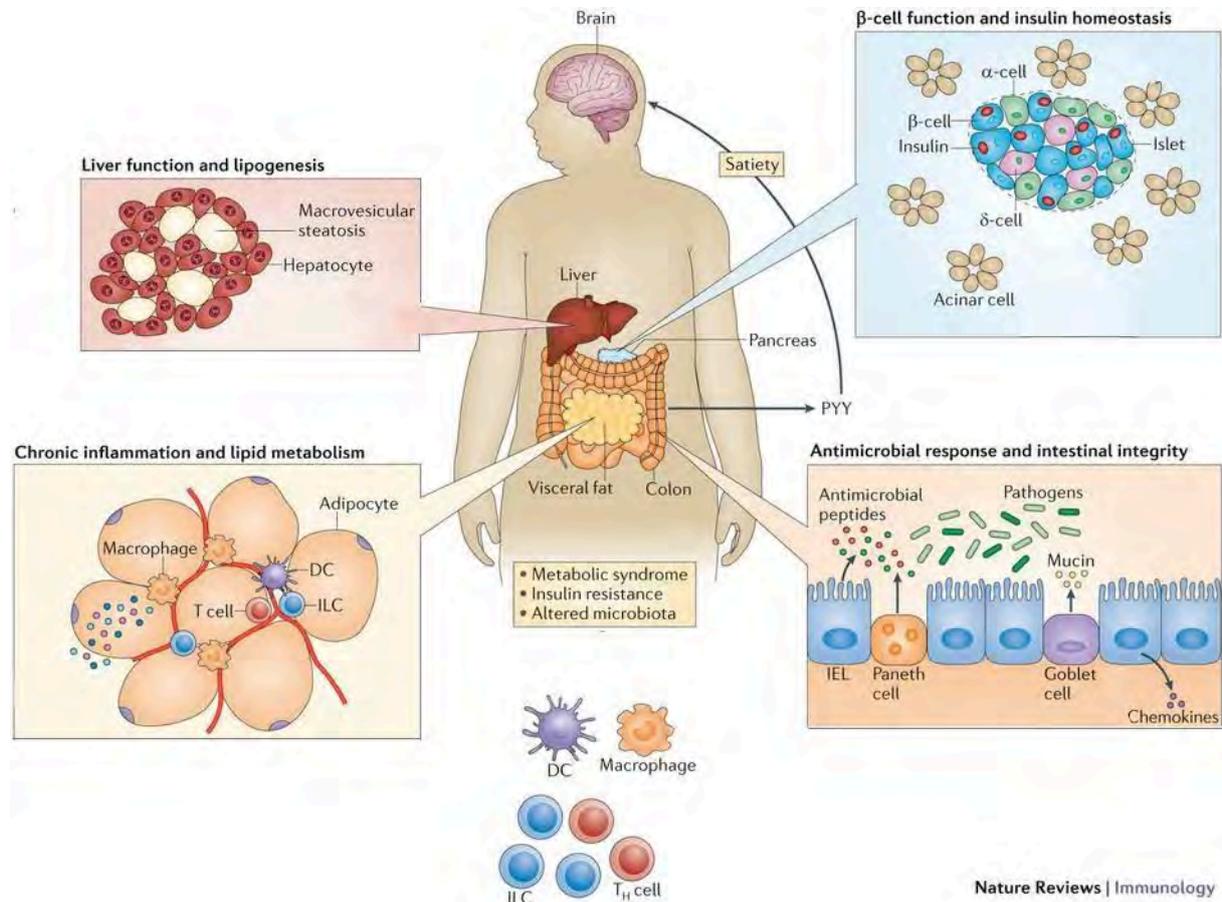


Figure 18 Disturbed body systems in metabolic disorders. Metabolic disorders are characterized chronic inflammation of the adipose tissue and increased lipid metabolism. In liver, increased gluconeogenesis and modified lipogenesis lead to steatosis. Peripheral insulin resistance alters also β -cell homeostasis in the pancreas. In intestine modified antimicrobial response, microbiota dysbiosis and altered intestinal integrity are observed. Intestinal hormones such as PYY are also impacted in metabolic disorders, leading to modified satiety signalling. Systemic inflammation is another hallmark of metabolic disorders. Figure modified from (Rutz et al., 2014).

Metabolic syndrome “is the name given to the aggregate of clinical conditions comprising central and abdominal obesity, systemic hypertension, insulin resistance (or type 2 diabetes mellitus), and atherogenic dyslipidemia” (McCracken et al., 2018). Type 2 diabetes mellitus (T2D) is a common metabolic disorder characterized by glucose intolerance and insulin resistance. “Insulin resistance is defined clinically as the inability of a known quantity of exogenous or endogenous insulin to increase glucose uptake and utilization in an individual as much as it does in a normal population” (Lebovitz, 2001). Insulin action is mediated by its binding on insulin receptor (IR) and subsequent cascade signaling. MAPK (pathway for mitogenesis) and PI3K (pathway for glucose metabolism regulation) are activated via IRS-1 and IRS-2. Various factors can interfere with elements of the signaling cascade and therefore

inhibit insulin action in the cell. Among them are cytokines and free fatty acids, which accumulate in inflammatory state and increased adipose tissue (for review: (Guo, 2014)).

The metabolic syndrome is underlining the fact that obesity, insulin resistance, dyslipidemia and hypertension are tightly linked. They have shared hallmarks, whereas not every single appear in the isolated pathology. Indeed, chronic systemic and local inflammation (adipose tissue, liver), intestinal barrier dysfunction, insulin resistance and disturbed insulin homeostasis, microbiota dysbiosis have been observed in metabolic syndrome (**Figure 18**). Thus, the set of metabolic diseases is associated with several complications. Patients with metabolic syndrome have faster developing atherosclerosis and increased risk for cardiovascular diseases due to high blood pressure and the increased circulating triglycerides and lipoproteins. The risk for chronic kidney disease is also increased in patients suffering from metabolic syndrome (McCracken et al., 2018).

There are different models to study metabolic disorders like T2D and obesity. First, there is diet-induced metabolic disorder: animals receiving high-caloric, which could be high-fat (HFD) or high-fat high sugar diet (HFHSD). Second, there are genetically induced metabolic disorders. In particular *ob/ob* mice – deficient for leptin, a satiety hormone, and *db/db* mice – deficient for leptin receptor. Both leading to the same phenotype: severe obesity due to hyperphagia and T2D. Obesity and T2D are often associated in animal models, thus obesity and diabetes will be treated together in the following paragraphs.

3. Incidence of metabolic disorders

Metabolic disorders are worldwide epidemic problems and as such a growing global challenge. The World Health Organization estimates that worldwide obesity has nearly doubled since 1980. In 2012, 3.7 million deaths were related to higher-than-optimal blood glucose and diabetes. In 2014, 422 million adults were diabetic (T1D and T2D) (World Health Organization, 2016). Blood hyperglycemia was ranked as 5th global risk factor for DALYs (disability-adjusted life-years) (Forouzanfar et al., 2015). Until recently, only adults were affected, but now children are becoming more often diabetics. Even if the signification of metabolic syndrome and T2D are increasing, the understanding of those pathologies is still unsatisfactory and preventive and therapeutic strategies still fail to face this epidemy.

Metabolic disorders are multifactorial diseases involving genetic and environmental factors. Among environmental factors, dietary habits such as overnutrition and western diet (high fat and high carbohydrates contents) as well as sedentarity associated with a reduction of

physical activity, are major contributors in metabolic diseases development (Freedman et al., 1999; Wing et al., 2001). Beside diet and life style habits, epidemiological studies highlighted an association between post-traumatic stress disorder (PTSD) (Agyemang et al., 2012; Goodwin and Davidson, 2005; Lukaschek et al., 2013) or adverse childhood experience (Alastalo et al., 2009; Huang et al., 2015) and T2D incidence.

4. Link between metabolic disorders and microbiota

Over the last decade, several studies have linked intestinal microbiota to the development of metabolic disorders. Along with genetic and environmental susceptibilities, the intestinal microbiota could trigger impairment in the energy homeostasis leading to diseases. Several studies, showed that intestinal microbiota is a signature of metabolic disorders (Amar et al., 2011; Bäckhed et al., 2004; Hotamisligil, 2006).

Genetically obese (*ob/ob*) mice had less Bacteroidetes and more Firmicutes than their lean siblings (Ley et al., 2005). In diet-induced obesity (HFD 60%) similar changes in the ratio of Bacteroidetes and Firmicutes were observed (Guo et al., 2017). A change in the ratio of those major phyla (Bacteroidetes to Firmicutes) leads to a new microbiota with an increased capacity to digest dietary fibers to produce monosaccharaides and short-chain fatty acids (SCFA) able to be absorbed by the host. Thus, microbiota has an impact on the energy harvest of the host (Turnbaugh et al., 2006).

This is not surprising as microbiota has a role on host metabolism in physiological conditions. Indeed, intestinal microbiota could down-regulate the production of fat-induced adipocyte factor (FIAF) by the intestinal cells, which in turn inhibits the activity of the lipoprotein lipase. These results are in agreement with other studies showing that GF mice fed HFD are resistant to diet-induced metabolic disorders and gain less weight than their conventional counterparts (Bäckhed et al., 2007; Nicholson et al., 2012). GF mice genetically lacking FIAF are not protected from diet-induced obesity (Bäckhed et al., 2007). Colonization of GF mice showed a dramatic increase in body fat within 10-14 days, despite an associated decrease in food consumption after colonization in comparison with GF state (GF mice are described to be hyperphagic) (Bäckhed et al., 2004). In addition, mice colonized with microbiota from obese mice gained more weight than those transferred with microbiota from lean mice, demonstrating a causal role of microbiota in the development of metabolic diseases (Turnbaugh et al., 2006).

In human, 16S ribosomal RNA sequencing of microbiota of obese individuals have been shown to be characterized by increased Firmicutes and decreased Bacteroidetes. This obese microbiota phenotype could be reversed by dietary interventions (Ley et al., 2005). Serino et al. observed that caecal microbiota is distinct between insulin resistant and insulin sensitive obese patients (Serino et al. 2012). Microbiota dysbiosis was observed in T2D patients (Qin et al., 2012). Microbiota of T2D patients and healthy control were distinct, even after stratification for metformin treatment (Forslund et al., 2015). In patients suffering from metabolic syndrome, microbiota transfer of lean donors improves hepatic insulin sensitivity 6 weeks after transfer (Vrieze et al., 2012). The integrative Human Microbiome Project (iHMP) aimed to focus on the role of microbiota on the onset of diseases including type 2 diabetes (T2D) (Zhou et al., 2019). The strength of iHMP in T2D is the following of participants (healthy and pre-diabetics) for four years (Zhou et al., 2019). This allows to associate microbiome profile to insulin sensitivity status that confirm microbiota dysbiosis associated to T2D and might contribute to early detection of T2D (Zhou et al., 2019).

Microbiota could trigger metabolic syndrome via its ability to modify intestinal permeability and/or immune response. Microbiota dysbiosis could lead to intestinal hyperpermeability and increased translocation of bacterial fragments that could reach the circulation.

5. The role of intestinal permeability in metabolic disorder

Indeed, intestinal hyperpermeability has been reported in metabolic disorders. In HFD-induced metabolic disorders in mice, intestinal permeability measured *in vivo* by FITC-Dextran 4 kDa (FD4) gavage was increased (Cani et al., 2008; Johnson et al., 2015). This was linked to reduced expression of genes coding for proteins of the tight junctions (Cani et al., 2008). Measurements in Ussing chambers confirmed increased intestinal permeability to FD4. Additionally, transepithelial conductance, an indicator for paracellular permeability, was also increased by HFD (Johnson et al., 2015). Of interest, Rohr et al wrote a complete review regarding the negative effects of HFD on intestinal permeability (Rohr et al., 2019).

Intestinal hyperpermeability is pointed out as a factor leading to translocation of bacterial products (Cani et al., 2007). It is found that plasma levels of bacterial lipopolysaccharides (LPS), components of the outer membranes of gram-negative bacteria, increase under HFD (Cani et al., 2007). Interestingly, LPS levels in mice are fluctuating in function of food intake, highlighting a potential link with intestinal barrier (Cani et al., 2007). LPS are highly inflammatogenic molecules (Schumann et al., 1990; Sweet and Hume, 1996;

Wright et al., 1990). Increased plasma LPS level is defined as metabolic endotoxemia. The increase is 10-50 times lower than values which could be reached during septicemia or other infections (Cani et al., 2007). Intestinal phagocytes (DC and macrophages) capture bacterial intestinal antigens, LPS, and transfer them into lysosomes for degradation (Sansone and Di Santo, 2007). These antigens activate CD14/TLR4 positive immune cells, which secrete cytokines contributing to low-grade inflammation. LPS can induce macrophage accumulation in white adipose tissue, but it is not essential for the impaired glucose metabolism associated with gut colonization (Caesar et al., 2012). Therefore, other substances besides the LPS could be responsible for the low-grade inflammation. The bacterial Peptidoglycan (PGN), piline, flagellin, fimbriae and bacterial DNA can also be transferred *via* intestinal barrier, taking para or transcellular route (Cani et al., 2007). LPS perfusion in mice under normal diet induced similar phenotype as HFD, namely fasted hyperglycemia, insulinemia, weight gain and increase in adipose tissue but also macrophage infiltration in adipose tissue and liver insulin resistance (Cani et al., 2007).

Some argue that even living bacteria could pass the intestinal barrier and enter into the organism. Using green fluorescent protein (GFP) labeled *Escherichia coli* (*E. coli*), it has been shown that *E. coli* GFP accumulated after gavage in mucosa in HFD-fed mice. Bacteria are co-localized with DC in intestinal *lamina propria* and probably disseminated inside DC towards mesenteric adipose tissue and mesenteric lymph nodes (MLN) via blood (Amar et al., 2011). However, these observations could rather be bacterial fragments or only GFP tracker, translocated into the circulation than living bacteria.

Another indicator of intestinal hyperpermeability in metabolic disease are increased levels of Ig directed against microbiota. HFD-fed mice exhibit increased IgG against *E. coli* in plasma (Mohammed et al., 2012)

Data on intestinal permeability in genetically obese mice are conflicting. Stenman et al. evaluated intestinal permeability in Ussing chambers using FD4 and did not find any modification in *ob/ob* vs WT (Stenman et al., 2013). However, Johnson described increased intestinal permeability to FD4 *in vivo* and *ex vivo* in Ussing chambers in *ob/ob* mice. Transepithelial conductance (paracellular permeability) was not different in comparison to WT (Johnson et al., 2015). Another study confirms these observations: Ye et al. observed increased intestinal permeability to FD4 *in vivo* in *ob/ob* mice in comparison to WT (Ye et al., 2018).

Data on intestinal hyperpermeability in metabolic disorders in human are similar to observations in mouse models. Increased intestinal permeability measured *in vivo* by lactulose/mannitol absorption is positively correlate with HOMA index (measure of insulin resistance) in obese patients (Teixeira et al., 2012). Additionally, LPS is elevated in plasma of apparently healthy men at risk of becoming obese and diabetic. Elevated level of LPS is correlated with energy intake (Amar et al., 2008). Furthermore, IgG against *E.coli* is increased in obese patients (Mohammed et al., 2012).

Dysfunction of the intestinal barrier and translocation of bacterial products could be at the origin of inflammation at local or systemic levels.

6. Low-grade inflammation in metabolic disorders

As already mentioned, it became clear that inflammation is a key feature of obesity and T2D (Hotamisligil, 2006; Pickup and Crook, 1998). However, one has to redefine the term of inflammation. Initially inflammation was described by using the following four characteristics: calor, rubor, tumor and dolor (fever, redness, swelling and pain) (Larsen and Henson, 1983). Then, metabolic disorders are characterized by a low-grade inflammation which triggers insulin resistance (Duncan et al., 2003; Pradhan et al., 2001; Shoelson et al., 2006). Low-grade inflammation is a state of chronic, but low-grade, secretion of inflammatory mediators, as for example cytokines. Indeed, levels of inflammatory mediators in low-grade inflammation are only slightly increased in comparison to healthy state.

The pro-inflammatory cytokine TNF- α plays also a role in the development of insulin resistance. Indeed, TNF- α increases lipolysis in adipocytes of rats (Green et al., 1994) and human (Zhang et al., 2002), thus increasing circulation fatty acids which is detrimental for insulin sensitivity. TNF- α neutralization has been shown to ameliorate insulin sensitivity in obese *fa/fa* rats (mutation in the gene for leptin receptor) (Hotamisligil et al., 1993). The excess of pro-inflammatory cytokines impairs cellular insulin signaling by increasing serine phosphorylation of insulin receptor substrate-1 (IRS-1) and thus contributes to insulin resistance and T2D (Feinstein et al., 1993; Hotamisligil et al., 1996). IL-6 provokes hypertriglyceridemia *in vivo* by stimulating lipolysis and hepatic triglyceride secretion in rats (Nonogaki et al., 1995).

Immune cells are also important for triggering of diseases. Innate and adaptive immune cells contribute to disease. During obesity and T2D, macrophages and lymphocytes infiltrate the adipose tissue and the liver. The accumulation of these immune cells is directly proportional

to measures of adiposity (adipocyte size, BMI) in mice and humans (Hotamisligil, 2006; Weisberg et al., 2003).

In mice, which had HFD-induced metabolic disorders and in *ob/ob* mice increased numbers of macrophages and CD8⁺ T lymphocytes were observed in the stromal fraction of epididymal fat pads in comparison to control mice under normal diet. In contrast, CD4⁺ T lymphocytes and Treg were significantly decreased (Nishimura et al., 2009). Additionally, mice under HFD, had increased secretion of IFN- γ . Kinetic analysis of adipose tissue infiltration following diet-induced insulin resistance have shown that infiltration by CD8 T cells preceded the accumulation of macrophages. CD8⁺ T cell elimination reduced macrophage infiltration and adipose tissue inflammation and ameliorated metabolic parameters (Nishimura et al., 2009).

In epidemiological and case-control studies in human, it has been shown that pro-inflammatory cytokines and biomarkers, like C-reactive protein (CRP) and IL-6, are increased in metabolic disorders (Duncan et al., 2003; Pradhan et al., 2001). Additionally, macrophages have been shown to accumulate in adipose tissue of obese patients (Weisberg et al., 2003).

7. Type 1 diabetes mellitus

Type 1 diabetes mellitus (T1D) is an autoimmune disorder with genetic predisposing factor accounting for more than 90% and hence in contrast to most of metabolic disorders, not due to inappropriate lifestyle. However, every person with genetic predisposition for T1D does not develop T1D suggesting not only a role for genetic but also environment. T1D is characterized by blood hyperglycemia due to a loss of insulin. Indeed, pancreatic β -cells are destructed by autoreactive T cells. A role of intestinal hyperpermeability in the development and/or course of disease is questioned.

During my PhD, Dr. Sandrine Ménard and myself were invited by Dr. Julien Diana, guest editor of the Journal of Frontiers in Immunology, to write a review about the intestinal barrier and stress in the case of autoimmune disorders: “Psychological Stress, Intestinal Barrier Dysfunctions and Autoimmune Disorders” submitted in May 2019 and at the moment under review. We also discuss the role of intestinal microbiota, intestinal permeability and immune response in the case of T1D. Parts of this review are reused in the following chapter; the review article itself is inserted at the end of chapter III.

CHAPTER III

STRESS

Stress, firstly described in 1936 by Selye, is defined as a real (physical) or perceived (psychological) threat to homoeostasis, to which the organism has to react by an adaptive response (Selye, 1936).

Lifetime window, length and frequency of exposure to stress play a pivotal role in consequences of stress on the individual pathophysiology. Indeed, acute and chronic stress exposure could occur in early life in a still maturing organism or at adulthood in mature organism. Traumatic experiences can lead to so called post-traumatic stress disorder (PTSD), a condition in which a person is suffering from anxiety, depression and flashbacks long after the traumatic experience (Kessler et al., 2005). Persisting stress or inadequate response can lead to harmful maladaptive reactions depending on the kind of stress.

The stress response is orchestrated by hypothalamo-pituitary-adrenal axis (HPA) and sympathetic nervous system (SNS). The neuroendocrine and autonomous response is mediated by hormones as epinephrine, norepinephrine, CRF, ACTH, glucocorticoids (cortisol in human and corticosterone in rodents) (Charmandari et al., 2005; Smith and Vale, 2006).

1. The HPA axis

The Hypothalamo-Pituitary-Adrenal axis consists of three organs (hypothalamus, hypophysis (or pituitary gland) and adrenal glands) and the hormones CRF, ACTH and glucocorticoids. In response to stress, corticotropin releasing factor (CRF) is released by the paraventricular nucleus of hypothalamus into blood vessels leading to the anterior pituitary gland. Here, CRF is binding to corticotrop cells, which release in response adrenocorticotrophic hormone (ACTH) into systemic circulation. Via the blood stream, ACTH is attaining its principal target the adrenal cortex. ACTH stimulates the synthesis and release of glucocorticoids (cortisol in human; corticosterone in rodents). Glucocorticoids act at numerous sites via its intracellular glucocorticoid receptor (GR). The HPA response is finely regulated by retrocontrol: Glucocorticoid can bind to receptors in hippocampus, hypothalamus, hypophysis via its GR and downregulate CRF and ACTH release (**Figure 19**) (Smith and Vale, 2006).

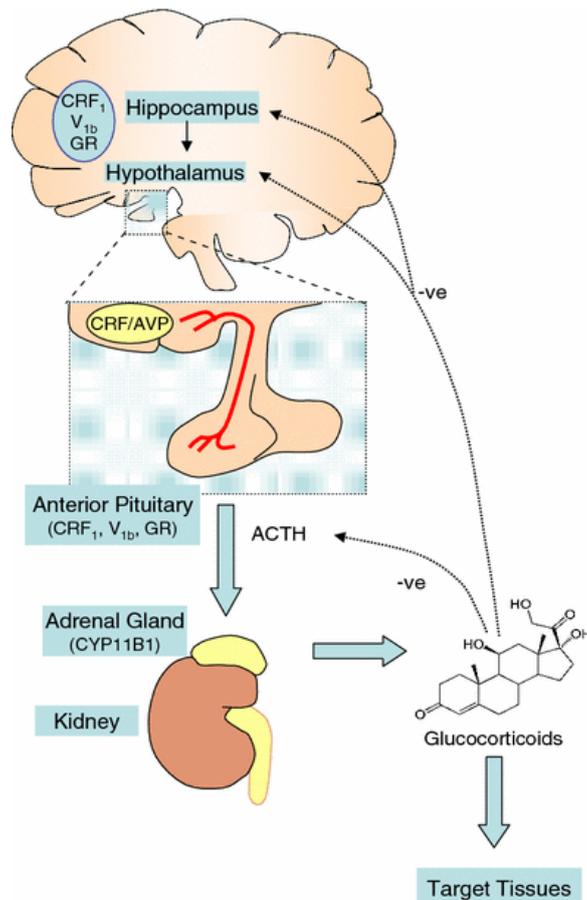


Figure 19 The hypothalamus-pituitary-adrenal axis. The hypothalamus is releasing CRF which reaches anterior pituitary gland stimulating the release of ACTH. ACTH acts on adrenal gland stimulating release of glucocorticoids. Glucocorticoids have various functions on diverse target tissues and can regulate via negative feedback CRF and ACTH release. CRF corticotropin releasing factor, ACTH Adrenocorticotropic hormone, AVP arginine vasopressin, V vasopressin, GR glucocorticoid receptor (Thomson and Craighead, 2008).

2. Immunomodulatory effect of glucocorticoids

A special attention has been paid to glucocorticoid in stress response and immune regulation. Endogenous glucocorticoids, part of the endocrine stress response, have ubiquitous functions in development, metabolism and inflammation. In general, glucocorticoids have been described to dampen immune response all along the inflammation process (for review (Cain and Cidlowski, 2017)). Glucocorticoids attenuate signaling pathways of many pattern recognition receptors (Beaulieu and Morand, 2011; Miyata et al., 2015), diminish leukocyte transmigration by reducing adhesion molecules (Atsuta et al., 1999). Glucocorticoids also decrease the production of chemoattractants (Mukaida et al., 1994), program macrophages to anti-inflammatory M2c subtype (high expression of scavenger receptors and secretion of anti-inflammatory cytokines) (Martinez et al., 2008) and decrease T cell response (Gillis et al., 1979a, 1979b) preferentially Th1 and Th17 by promoting Th2 and Treg (Elenkov, 2004; Liberman et al., 2007). Due to their immunosuppressive effects, glucocorticoids have been used to treat various immune related disorders like autoimmune disorders (AD).

The literature of the past 60 years focused on immunosuppressive properties of glucocorticoids but glucocorticoids can also enhance inflammation and immunity (for review (Cain and Cidlowski, 2017)). Part of the explanation of conflicting effects of glucocorticoid on immune response might reside in the diversity of glucocorticoid-receptors in different tissues, the presence or absence of 11 β HSD, an enzyme inactivating cortisol as well as the time of glucocorticoid exposure (before or after tissue injury/inflammation) (Frank et al., 2010) and the dose (Lim et al., 2007). As an example, in humans, childhood maltreatment is associated with modified methylation of the glucocorticoid receptor gene NR3C1 in adults in brain and in leucocytes (McGowan et al., 2009; Melas et al., 2013; Perroud et al., 2011). All those factors might explain that stressful events inducing glucocorticoids release play a role in AD occurrence that can be treated by exogenous glucocorticoids.

3. Effect of stress on intestinal barrier

Stress is playing a role in the course of gastro-intestinal disorders like irritable bowel syndrome (IBS) (Hislop, 1979; Lowman et al., 1987; Videlock et al., 2009) and inflammatory bowel diseases (IBD) (Sgambato et al., 2017). IBS is a very interesting model to study the consequences of stress on intestinal barrier. Indeed, the occurrence of stressful events is considered as a contributing factor triggering and/or maintaining IBS (Mayer et al., 2001; Wood, 2011), suggesting that dysfunctional interactions in the brain-gut axis contribute to the pathophysiology of the disease (Bonaz and Bernstein, 2013) and as such justifying its new classification as disorder of the brain-gut interaction (Drossman, 2016).

Stress comes along with microbiota modifications, intestinal and systemic inflammation and modified intestinal permeability in rodent and human. It is difficult to decipher if stress directly influences microbiota or if the observed effects are a consequence of modified immune response or intestinal physiology. These actors are tightly linked and regulate one another.

3.1 Stress affects microbiota composition

Neonatal maternal separation induced microbiota dysbiosis in mice (Riba et al., 2017). Limited nesting stress altered microbiota in rat pups (Moussaoui et al., 2017). In adults, chronic water avoidance stress increased susceptibility to indomethacin induced hyperpermeability in mice and the effect is transferable *via* fecal microbiota transfer (Yoshikawa et al., 2017). Germ free (GF) mice have exaggerated HPA stress response after restraint stress (Sudo et al., 2004) showing the role of microbiota in the regulation of stress response. Mice exposed to social disruption stress have increased circulating IL-6 and MCP-1 levels, these effects were totally

abolished by antibiotic mixture (ampicillin, vancomycin, neomycin sulfate, and metronidazole), showing the importance of microbiota in the induction of stress effects (Bailey et al., 2011). However, one cannot exclude an effect induced directly by the antibiotic treatment on the physiology of the intestine, and not by microbiota.

In humans, decreased total abundance of Actinobacteria, Lentisphaerae, and Verrucomicrobia is associated with PTSD in south Africans individuals (Hemmings et al., 2017). Microbiota dysbiosis has been described in IBS patients (for review (Collins, 2014)).

3.2 Stress affects intestinal permeability

In preclinical model and epidemiological studies, stress has been associated with an increase of intestinal permeability. Chronic water avoidance stress increases intestinal permeability and decreases tight junction protein expression in colon of adult rat (Zheng et al., 2013) and overall intestinal permeability in mice (Cameron, 2005). Chronic neonatal maternal separation, a model of early life stress, also increases intestinal permeability in rat (Barreau et al., 2004; Moussaoui et al., 2017; Øines et al., 2012) and male mice (Riba et al., 2018) but not female. Maternal separation applied just for one time (acute stress) increases intestinal permeability in rats, which has been shown being mediated by stress hormones glucocorticoids. Indeed, using GR inhibitor there were no increase in intestinal permeability after acute maternal separation (Moussaoui et al., 2014). Combination of different stressors (subacute (isolation, limited movement) and chronic crowding stress) also decreased tight junction mRNA expression in rats (Laufer et al., 2016). In a mouse model of social disruption, a social stressor, bacterial RNA (*Lactobacillus spp.*) is increased in spleen, which indicates translocation of at least bacterial fragments (Lafuse et al., 2017).

In human, acute psychological stress like public speaking has also been shown to induce intestinal hyperpermeability (Vanuytsel et al., 2014). Intestinal hyperpermeability has also been described in IBS patients, but only in IBS-diarrhea predominant patients (Bischoff et al., 2014). The stress hormone cortisol (human) and corticosterone (mice) have been shown to mediate stress increased intestinal hyperpermeability as administration of the GR agonist dexamethasone mimicks the intestinal hyperpermeability (Moussaoui et al., 2014; Zheng et al., 2013).

3.3 Stress modifies intestinal immune system

Chronic neonatal maternal separation in rats increases cytokine expression, myeloperoxidase activity and mast cell numbers in colonic tissue and exacerbate TNBS-

induced colitis (Barreau et al., 2004). Neonatal maternal separation in mice increased TNF α expression by intestinal tissue in young adult (Riba et al., 2017). Acute restraint stress augments histamine release by mast cells (Eutamene et al., 2003). Acute acoustic stress increased intestinal IL-17 and IL-22 expression in mice (Miranda and Roux, 2017).

In human, stress aggravates IBD symptoms including higher release of pro-inflammatory effectors (Sgambato et al., 2017). In IBS, an increased state of activation of immune cells has been described even though this observation is under debate (Barbara et al., 2011).

3.4 Stress alters systemic immune response

Not only the intestinal immune system is influenced by psychological stress. There is also evidence for modified systemic immune response without direct proof that inflammatory immune cells were activated in gastrointestinal tract. Neonatal maternal deprived rats have increased cytokine expression in liver and spleen (Barreau et al., 2004). Humoral immune response against microbiota is increased in neonatal maternal deprived mice (Riba et al., 2018). Social disruption stress in mice increases bacterial translocation and induces circulating IL-6 and MCP-1 (Bailey et al., 2011; Lafuse et al., 2017).

Eutamene et al. showed that acute stress-induced increase of histamine content in mast cells of rats was mediated by IL-1 β and CRF. Injection of both individually mimics stress-induced effects and inhibition of IL-1 β blocked stress-induced mast cell changes (Eutamene et al., 2003). These findings are highlighting the close relationship and interaction between HPA, intestinal and systemic immune response.

Stress is associated with an increase in pro-inflammatory response as described in PTSD patients (de Oliveira et al., 2018). A meta-analysis of several studies showed that IL-6, TNF α and IL-1 β secretion were increased in response to acute stress in human (Marsland et al., 2017). Childhood victimization is associated with elevated CRP levels in young adult (Baldwin et al., 2017; Slopen et al., 2015).

3.5 Visceral sensitivity

Stress has also been described to induce visceral sensitivity. “Hypersensitivity refers to increased sensation of stimuli. In practice, this is appraised by measurement of threshold volumes or pressures for first sensation or pain. Alternatively, it refers to the increased scores of symptoms (including pain) in response to standard stimuli. Hyperalgesia refers to increased

pain sensation in response to a certain stimulus. Allodynia refers to the appreciation that a stimulus, which was previously not perceived as being painful, becomes painful.” (Camilleri et al., 2001).

Acute water avoidance stress (1h) induces transient visceral hyperalgesia, this effect was mediated by GR (Myers and Greenwood-Van Meerveld, 2012). Partial restraint stress (2h) induces hypersensitivity in response to rectal distension in rats (Gué et al., 1997). Chronic maternal separation leads to visceral hypersensitivity in young adult male and female mice (Riba et al., 2018, 2017). In studies with rodents, it has been shown, that the modified pain perception by stress is due to modifications of the HPA. Indeed, the effects have been shown to be mediated by GR (Myers and Greenwood-Van Meerveld, 2012) and could be mimicked by CRF injection (Gué et al., 1997) but also inflammation could influence pain perception since mast cell numbers, IL-1 β and IFN γ are increased by chronic water avoidance stress (Bradesi et al., 2005; Gué et al., 1997).

In human, IBS patients are often suffering from increased visceral sensitivity (Ludidi et al., 2012; van der Veek et al., 2008). Stressful life events and early childhood adverse experiences are described to aggravate pain in IBS patients (Lampe et al., 2003).

4. Effects of stress on glucose metabolism

Glucocorticoids have a direct influence on metabolism. Indeed, in acute stressful situations stress hormones stimulate catabolic processes in order to maintain life. Glucose and free fatty acids are distributed to muscles in order to prepare a “fight or flight” response. However, acute and chronic stress do not have the same impact on metabolism. Whereas acute stress leads to feeding suppression and body weight loss, chronic stress is associated with food overconsumption and body weight gain (Rabasa and Dickson, 2016). Stress has also an impact on insulin sensitivity. Acute chronic stress in mice induced by inescapable food shocks led rapidly to insulin resistance (Li et al., 2013). In human, psychosocial stress –related variables were associated with hyperglycemia, hyperinsulinemia and increased plasminogen activator inhibitor-1 antigen comprising the IRS, even after adjusting for BMI (Räikkönen et al., 1996).

In this chapter, I discussed the effect of stress on intestinal barrier and glucose metabolism. There is also tremendous data about a link between stress and autoimmune disorders. During my PhD, Dr. Sandrine Ménard and myself were invited by Dr. Julien Diana, guest editor of the Journal of Frontiers in Immunology, to write a review about the intestinal

barrier and stress in the case of autoimmune disorders: “Psychological Stress, Intestinal Barrier Dysfunctions and Autoimmune Disorders” submitted in May 2019 and at the moment under review.

Psychological Stress, Intestinal Barrier Dysfunctions and Autoimmune Disorders

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7 **Keywords: Intestinal Permeability, Psychological Stress, Type 1 Diabetes, Multiple Sclerosis,**
8 **Systemic Lupus Erythematosus, Microbiota, Immune Response.**

9 **Abstract**

10 **Autoimmune disorders (AD)** are multifactorial diseases involving, genetic, epigenetic and
11 environmental factors characterized by an inappropriate immune response toward self-antigens. In the
12 past decades there has been a continuous rise in the incidence of AD which cannot be explained by
13 genetic factors alone. Influence of **psychological stress** on the development or the course of
14 autoimmune disorder is debated for a long time. Indeed, based on epidemiological studies, stress has
15 been suggested to precede AD occurrence and to exacerbate symptoms. Furthermore, compiling data
16 showed that most of AD are associated with **gastro-intestinal symptoms** i.e. microbiota dysbiosis,
17 intestinal hyperpermeability and intestinal inflammation. Interestingly, social stress (acute or chronic,
18 in adult or in neonate) is a well described intestinal disrupting factor. Taken together those observations
19 questioned a potential role of stress-induced defect of intestinal barrier in the onset and/or the course
20 of AD.

21 In this review, we aim to present evidences supporting a role of stress-induced intestinal barrier
22 disruption in the onset and/or the course of AD. We will mainly focus on auto-immune Type 1
23 Diabetes, Multiple Sclerosis and Systemic Lupus Erythematosus, AD for which we could find sufficient
24 circumstantial data to support this hypothesis.

25 **1 Introduction**

26 Autoimmune disorders (AD) are multifactorial diseases involving, genetic, epigenetic and
27 environmental factors. In the past decades there has been a continuous rise in the incidence of AD
28 which cannot be explained by genetic factors alone. Changes in our lifestyle including, diet, hygiene,
29 exposure to social adversity or pollutants have been suggested to be risk factors for AD. AD are
30 associated with defect of intestinal barrier and besides nutrition, one other environmental factor well
31 described to impair intestinal barrier is psychological stress. The aim of this review is to compile
32 evidences highlighting a relationship between stress, intestinal barrier disruption and occurrence of
33 AD. Even though, there are no striking studies linking stress-induced intestinal barrier defect to AD
34 onset, our goal is to combine evidences based on a review of the literature and offer a new field of
35 research and perspectives on AD. We will focus on three of the most studied AD: auto-immune type 1
36 diabetes (T1D), systemic lupus erythematosus (SLE) and multiple sclerosis (MS).

37 This review is based on epidemiological and pre-clinical studies. Numerous excellent and recent
38 reviews treating either AD and stress or stress and intestinal barrier will be quote to support this
39 hypothesis.

40 **2 Stress**

41 Stress, firstly described in 1936 by Selye, is defined as a real (physical) or perceived (psychological)
42 threat to homeostasis, to which the organism has to react by an adaptive response (1).

43 Life time window, length and frequency of exposure to stress play a pivotal role in consequences of
44 stress on the individual pathophysiology. Indeed, acute and chronic stress exposure could occur in early
45 life in a still maturing organism or at adulthood in mature organism. Traumatic experiences can lead
46 to so called post-traumatic stress disorder (PTSD), a condition in which a person is suffering from
47 anxiety, depression and flashbacks long after the traumatic experience (2). Persisting stress or
48 inadequate response can lead to harmful maladaptive reactions depending on the kind of stress that will
49 be discussed below.

50 The stress response is orchestrated by hypothalamic pituitary adrenal axis (HPA) and sympathetic
51 nervous system (SNS). The neuroendocrine and autonomous response is mediated by hormones as
52 epinephrine, norepinephrine, CRH, ACTH, glucocorticoids (cortisol in human and corticosterone in
53 rodents) (3,4). A special attention has been paid to glucocorticoid in stress response and immune
54 regulation. Endogenous glucocorticoids, part of the endocrine stress response, have ubiquitous
55 functions in development, metabolism and inflammation. In general, glucocorticoids have been
56 described to dampen immune response all along the inflammation process (for review (5)): they
57 attenuate signaling pathways of many pattern recognition receptors (6,7), diminish leukocyte
58 transmigration by reducing adhesion molecules (8), decreasing the production of chemoattractants (9),
59 program macrophages to anti-inflammatory M2c subtype (high expression of scavenger receptors and
60 secretion of anti-inflammatory cytokines) (10), decrease T cell response (11,12) preferentially Th1 and
61 Th17 by promoting Th2 and Treg (13,14). Due to their immunosuppressive effects, glucocorticoids
62 have been used to treat various immune related disorders like AD.

63 The literature of the past 60 years focused on immunosuppressive properties of glucocorticoids but
64 glucocorticoids can also enhance inflammation and immunity (for review (5)). We will not go into the
65 details of conflicting effects of glucocorticoid on immune response but part of the explanation might
66 reside in the diversity of glucocorticoid-receptors in different tissues, the presence or absence of
67 11 β HSD, an enzyme inactivating cortisol as well as the time of glucocorticoid exposure (before or after
68 tissue injury/inflammation) (15) and the dose (16). All those factors might explain that stressful events
69 inducing glucocorticoids release play a role in AD occurrence that can be treated by exogenous
70 glucocorticoids. As an example, in humans, childhood maltreatment is associated with modified
71 methylation of the glucocorticoid receptor gene NR3C1 in adults in brain and in leucocytes (17–19).

72 **3 Role of stress in AD (for rev (20))**

73 The onset of at least 50% of autoimmune disorders has been attributed to unknown trigger factors.
74 Many retrospective studies observed that that most of patients suffering from AD report uncommon
75 emotional stress before disease onset (21). This is obviously a vicious cycle as AD diseases causes
76 stress in patients (22,23). This review will focus on three of the most studied AD that will provide
77 sufficient evidence to support the hypothesis of a role of stress-induced intestinal barrier defect on AD
78 onset i.e. T1D, SLE and MS. **Type 1 Diabetes** (T1D) is an organ specific AD characterized by

79 autoimmune response against host pancreatic β -cells leading to a defect of insulin production by
80 pancreas (24). **Systemic lupus erythematosus** (SLE) is an AD characterized by severe and persistent
81 inflammation that leads to tissue damage in multiple organs (25,26). **Multiple sclerosis** (MS) is a
82 chronic inflammatory disease of the central nervous system with a pathogenesis characterized by a
83 breakdown of the blood-brain barrier and demyelination of the central nervous system by infiltrating
84 auto-reactive T cells (27,28). The most widely used preclinical MS model is Experimental
85 Autoimmune Encephalomyelitis (EAE).

86 A potential association between stressful events and **T1D** has been highlighted already longtime ago
87 when Thomas Willis links, in the 17th century, T1D onset to prolonged sorrow (29). Early life stress
88 seems to be of particular risk for T1D development (30,31). This is in accordance with literature
89 highlighting neonatal maturation of pancreas as critical and vulnerable to stressors (32). Stress in adult
90 has been described to increase incidence of **SLE** (33) and is able to exacerbate SLE symptoms (physical
91 pain, sleep disturbances and unemployment) (34). Around 70% of **MS** patients reported unusual
92 amount of stress before the onset of the disease (35,36).

93 Those epidemiological studies suggest that stress could be involved in both triggering or exacerbating
94 AD. Whether it is dependent on the kind of stress or AD involved is unknown and it would be
95 interesting to conduct both retrospective epidemiological studies and preclinical studies to better
96 document the role of stress in AD. However, some interventional studies suggest that stress
97 management could benefit to AD patients. Indeed, escitalopram (antidepressant) decreases the risk of
98 MS relapsing in women (37). Diazepam (tranquilizer) decreases EAE incidence and histological signs
99 associated with this disease in a mice model (38). A meta-analysis of ten randomized controlled trials
100 in children and adolescents showed that supportive or counseling therapy, cognitive behavioral therapy
101 and family system therapy reduce glycosylated hemoglobin and as such improve diabetes control (39).
102 Interestingly, in 11 studies in adults no beneficial effect of stress management could be observed on
103 T1D (39).

104 **4 Consequences of stress on intestinal barrier and systemic immune response**

105 Stress can affect various physiological process. Already Selye observed and others confirmed that the
106 gastrointestinal tract and the immune system are among particularly responsive to stress no matter the
107 origin of the stress (1).

108 **4.1 Actors of intestinal barrier and function**

109 Intestinal epithelium is the mammalian organism's biggest surface in contact with the environment.
110 Therefore, intestinal barrier function is highly diverse and well developed. The intestinal barrier have
111 to fulfill conflicting functions. Indeed, intestinal barrier allows the transport of nutrient but at the same
112 time filters and defends the organism from harmful luminal content (pathogens, toxins...). Among the
113 main actors of intestinal barrier we can quote, intestinal microbiota, intestinal epithelium and immune
114 response (innate and adaptive). All those actors are in close relationship and regulate one another (for
115 review (40)). Not only intestinal microbiota participates in the protection against pathogens
116 colonization but also contributes to maturation of intestinal epithelium and immune system and
117 provides various nutritional compounds (41). The intestinal epithelium is formed by distinct cell types
118 distributed along the crypt-villus axis. Although they all derived from a common stem cell progenitor
119 located in the crypts, their morphology and roles differ (for review (42)). The intestinal epithelium is
120 renewed every five days and this constant renewing confers high plasticity and protection to the
121 intestinal barrier since defective cells are removed rapidly (43). Intestinal permeability is the ability of
122 intestinal epithelium to allow the selective entrance of luminal antigens into the organism (44). Another

123 actor of the intestinal barrier is the intestinal immune system (Gut associated lymphoid tissue – GALT)
124 harbored in the *lamina propria* and the Peyer patches (PP). GALT comprises the PPs, the appendix
125 and isolated lymphoid follicles (ILFs), which are considered inductive sites for mucosal B and T cells.
126 The humoral response in the intestine can be divided in four stages: predominant IgA induction in
127 mucosal B cells, recirculation of IgA plasma blasts and homing into the intestinal mucosa, terminal B
128 cell differentiation to plasma cells with local IgA production and export of IgA through the intestinal
129 epithelial layer (for review (45)). Most intestinal T cells mature in peripheral lymphoid organs. These
130 cells gain the expression of intestinal homing receptors to migrate to the intestine. Intestinal
131 lymphocytes are continuously exposed to food and microbial antigens. These lymphocytes help
132 maintaining the integrity of the intestinal barrier and immune homeostasis. Due to their close location
133 to luminal antigens they have both regulatory and effector capabilities, including the prevention of
134 pathogenic invasion and maintenance of tolerance to prevent extensive tissue damage (for review (46)).
135 Innate lymphoid cells (ILCs) are lymphocytes that do not express the type of diversified antigen
136 receptors expressed on T cells and B cells. ILCs are largely tissue-resident cells participating in tissue
137 homeostasis (for review (47)). A defective intestinal barrier will lead to inappropriate intestinal but
138 also systemic immune response leading to gastrointestinal disorders but also to extra intestinal diseases
139 like autoimmune diseases (48–50).

140 Intestinal barrier homeostasis is highly regulated and a defect in microbiota composition could lead to
141 intestinal hyperpermeability and intestinal inflammation. Intestinal inflammation will not only
142 contribute to intestinal hyperpermeability (51) but will also favor microbiota colonization by
143 pathobionts (52).

144 **4.2 Psychological stress impairs intestinal barrier**

145 Stress is playing a role in the course of gastro-intestinal disorders like irritable bowel syndrome (IBS)
146 (53–55) and inflammatory bowel disease (IBD) (56). IBS is a very interesting model to study the
147 consequences of stress on intestinal barrier. Indeed, the occurrence of stressful events is considered as
148 a contributing factor triggering and/or maintaining IBS (57,58), suggesting that dysfunctional
149 interactions in the brain-gut axis contribute to the pathophysiology of the disease (59) and as such
150 justifying its new classification as disorder of the brain-gut interaction (60). In this review, we will
151 focus on the consequences of psychological stress on intestinal barrier and its consequences on
152 systemic immune response.

153 **4.2.1 Microbiota dysbiosis**

154 Stress is modifying microbiota in animal and human. Neonatal maternal separation induced microbiota
155 dysbiosis in mice at different ages (61,62). Limited nesting stress altered microbiota in rat pups (63).
156 In adults, chronic water avoidance stress increased susceptibility to indomethacin induced
157 hyperpermeability in mice and the effect is transferable *via* fecal microbiota transfer (64). Germ free
158 (GF) mice have exaggerated HPA stress response after restraint stress (65) showing the role of
159 microbiota in the regulation of stress response. Mice exposed to social disruption stress have increased
160 circulating IL-6 and MCP-1 levels, these effects were totally abolished by antibiotic treatment showing
161 the importance of microbiota in the induction of stress effects (66).

162 In humans, decreased total abundance of Actinobacteria, Lentisphaerae, and Verrucomicrobia is
163 associated with PTSD in south Africans individuals (67). Microbiota dysbiosis has been described in
164 IBS patients (for review (68)).

165 **4.2.2 Stress is associated with intestinal hyperpermeability**

166 In preclinical model and epidemiological studies stress has been associated with an increase of
167 intestinal permeability. Chronic water avoidance stress increases intestinal permeability and decreases
168 tight junction protein expression in colon of adult rat (69) and overall intestinal permeability in mice
169 (70). Chronic neonatal maternal separation, a model of early life stress, also increases intestinal
170 permeability in rat (63,71,72) and mice (73). Maternal separation applied just for one time (acute stress)
171 increases intestinal permeability in rats (74). Combination of different stressors (subacute (isolation,
172 limited movement) and chronic crowding stress) also decreased tight junction mRNA expression in
173 rats (75). In a mouse model of social disruption, a social stressor, bacterial RNA (*Lactobacillus spp.*)
174 is increased in spleen, which indicates bacterial translocation (76).

175 In human, acute psychological stress like public speaking has also been shown to induce intestinal
176 hyperpermeability (77). Intestinal hyperpermeability has also been described in IBS patients (78). The
177 stress hormone cortisol (human) and corticosterone (mice) have been shown to mediate stress increased
178 intestinal hyperpermeability as administration of the GR agonist dexamethasone mimicks the intestinal
179 hyperpermeability (69,74).

180 **4.2.3 Stress exacerbates Intestinal and systemic inflammation**

181 Chronic neonatal maternal separation in rats increases cytokine expression, myeloperoxidase activity
182 and mast cell numbers in colonic tissue and exacerbate TNBS-induced colitis (71). Neonatal maternal
183 separation in mice increased TNF α expression by intestinal tissue in young adult (61) and LPS-
184 stimulated TNF α secretion of isolated *lamina propria* immune cells in ageing (62). Acute restraint
185 stress augments histamine release by mast cells (79). Acute acoustic stress increased intestinal IL-17
186 and IL-22 expression in mice (80).

187 In human, stress aggravates IBD symptoms including higher release of pro-inflammatory effectors
188 (56). In IBS, an increased state of activation of immune cells has been described even though this
189 observation is under debate (81).

190 Not only the intestinal immune system is influenced by psychological stress. There is also evidence for
191 modified systemic immune response without direct proof that inflammatory immune cells were
192 activated in gastrointestinal tract. Neonatal maternal deprived rats have increased cytokine expression
193 in liver and spleen (71). Humoral immune response against microbiota is increased in neonatal
194 maternal deprived mice (62,73). Social disruption stress in mice increases bacterial translocation and
195 induces circulating IL-6 and MCP-1 (66,76).

196 Stress is associated with an increase in pro-inflammatory response as described in PTSD patients (82).
197 A meta-analysis of several studies showed that IL-6, TNF α and IL-1 β secretion were increased in
198 response to acute stress in human (83). Childhood victimization is associated with elevated CRP levels
199 in young adult (84,85).

200 **5 Defect of intestinal barrier in AD (for review (86))**

201 We provided evidence that stress might play a role in onset or course of AD and review the well-
202 documented deleterious role of stress in intestinal barrier functions. We will now summarize the data
203 regarding the defect of intestinal barrier in AD. Indeed, the observed defect of intestinal barrier in AD
204 is an interesting lead that largely contribute to the rise of the hypothesis suggesting a contribution of
205 stress induced intestinal barrier defect in AD.

206

5.1 Microbiota in AD

207 Microbiota is known to contribute to intestinal mucosal permeability, induction of innate defenses and
208 as such represent a risk factor for AD (87,88). A growing body of evidence suggests that intestinal
209 microbiota can modify the incidence and/or severity of immune-mediated extra-intestinal diseases
210 (89). Aberrant microbiota has been described in patients suffering from type 1 diabetes (T1D) (90)
211 systemic lupus erythematosus (SLE) (88) and multiple sclerosis (MS) (91). Knowledge regarding
212 microbiota dysbiosis in AD patients and animal models will be summarized here and interventional
213 studies helping to understand the role of microbiota in those diseases will be discussed at the end of
214 the paragraph.

215 Increased microbial diversity and decreased level of butyrate producing Clostridia have been found in
216 microbiota of pediatric **T1D** patients (92)(93). In the BABYDIET cohort, alteration in the composition
217 of mucin degrading bacteria, with increased Bacteroides and decreased abundance of *Akkermansia*,
218 associate with the risk of early development of islet auto-antibodies (94). Thus, changes in microbial
219 diversity and differences in relative abundance, highlight that gut microbiota and associated increased
220 gut permeability may contribute to disease onset or its progression. A reduction of microbial diversity
221 is more pronounced before the time of diabetes onset (95). Fecal transplantation of NOD diabetic
222 microbiota in NOD resistant mice resulted in insulinitis induction revealing a diabetogenic gut microbial
223 community (96,97). Antibiotic treatment accelerates disease development (98,99) suggesting a
224 protective role of microbiota colonization in T1D.

225 The presence of a disrupted or altered microbiota in relapsing remitting **MS** patient compared to
226 healthy control has been observed (100–103) with no consensus on the involvement of a particular
227 bacterial species. In contrast another study comparing 16S RNA profiles of feces from MS and healthy
228 patients did not show any differences (101). Demyelination initiates after colonization with feces of
229 specific pathogen free mice (104). Microbiota depletion by non-absorbable antibiotics ameliorates the
230 development of EAE by reducing the number of mesenteric Th17 cells (105). GF mice present
231 attenuated symptom in both spontaneous and induced EAE models (104,106) suggesting a deleterious
232 role of microbiota colonization in MS. Furthermore, microbiota shapes and predicts the course
233 (chronic-progressive or relapse remitting) of EAE in a mice model (107).

234 Only few studies on human **SLE** microbiome in small cohorts reported microbial dysbiosis (108–110)
235 confirmed by pre-clinical studies in mice models (110). A study performed in a larger and diversified
236 cohort of SLE patient showed that the severity of disease is associated with more severe microbiota
237 dysbiosis (111).

238 5.1.1 Interventional studies. What do they tell us?

239 Here, we will focus on direct supplementation by living bacteria like probiotic and fecal microbiota
240 transplantation (FMT) treatment but not indirect interventions like prebiotics or nutritional compounds
241 produced by bacteria, as short chain fatty acids for example, that may involve indirect effects. Once
242 AD are diagnosed, the production of antibody against self-antigen will remain and will still damage
243 tissues but this process could be delayed or reduced. Probiotics are living microorganisms which confer
244 a health benefit to the host (112). Probiotics are known to have beneficial effects on intestinal barrier
245 (113,114) and anti-inflammatory properties (115–119) and as such represent an interesting tool to
246 delay, reduce or even prevent AD.

247 Animal studies suggest beneficial effects of probiotics supplementation on **EAE** via a stimulation of
248 IL-10 production (106,120–124). Clinical studies showed that a mixture of probiotics improved

249 expanded disability status score and decreased inflammatory markers (125). Regarding **T1D**, probiotic
250 treatment delays the onset of T1D in an experimental rat model and improves intestinal barrier (126).
251 Probiotics also protects NOD mice from T1D by reducing intestinal inflammation (127). In humans, it
252 was demonstrated in the TEDDY (The Environmental Determinants of Diabetes in the Young) cohort
253 that early probiotic supplementation was associated with a decreased risk of islet autoimmunity
254 compared to late or missing supplementation (128). In animal models for **lupus** nephritis, probiotic
255 administration lowers inflammatory response in kidney and intestine in female and castrated males but
256 not in non-castrated males (129).

257 FMT with microbiota from different diabetes resistant mouse strains delayed the onset of **T1D** in NOD
258 diabetes prone mice (97). Few studies investigate to role of FMT on AD symptoms and most of the
259 time the recommendation for FMT treatment was to target associated gastrointestinal troubles.
260 Neurological symptoms were improved and disease progression was paused in three **MS** patients after
261 FMT treatment for chronic constipation (130). Unfortunately, no data are available on intestinal barrier
262 function of probiotics and FMT treatments in parallel of beneficial effects on AD.

263 **5.1.2 Mimicking**

264 Structural similarities between antigens from infectious agents and myelin proteins (molecular
265 mimicry) can induce activation of naïve autoreactive T cells which will recognize peptides derived
266 from both infectious agents and self-antigens. Cross reactivity could occur when important motifs are
267 conserved and overall structures of TCR-peptide-MHC interaction are similar suggesting that cross
268 reactivity may happen frequently (131). Myelin basic protein, the immunodominant autoantigen of
269 MS, cross react with Epstein Barr Virus (EBV), influenza A virus, herpes simplex virus, human
270 papilloma virus (132) or human herpesvirus-6 (133). Regarding EBV, MS patients seem to exhibit
271 higher antibody titers against certain antigenic components of the virus than control subjects even
272 before the onset of **MS** (134). Despite cross-reactivity, infectious agents can impair self-antigen
273 tolerance by indirect activation (135). It has been showed that an integrase expressed by intestinal
274 Bacteroides encodes a low-avidity mimotope of the pancreatic β cell autoantigens and as such might
275 participate to **T1D** onset. Colonization of GF mice with Bacteroides promotes the recruitment of
276 diabetogenic CD8⁺ T cells to the gut (136).

277 **5.2 Intestinal hyperpermeability in AD**

278 In AD, intestinal hyper-permeability has been described, resulting in an increased entry of luminal
279 antigens derived from food and/or intestinal microbiota or pathogens. The resulting inflammation has
280 been suggested to participate in AD onset and/or exacerbation. However, if intestinal
281 hyperpermeability is a cause or a consequence of inflammation is under debate. Furthermore, if the
282 microbiota dysbiosis is a cause or a consequence of intestinal hyperpermeability and inflammation is
283 still unclear.

284 Even though it is still unclear whether intestinal hyperpermeability is trigger or a consequence of **T1D**
285 progression (137,138)(93) epidemiological and pre-clinical studies demonstrated that intestinal
286 hyperpermeability occurs before disease onset (139,140). Reversion of intestinal hyperpermeability by
287 treatment with a zonulin 1 (intestinal homolog of an Vibrio cholerae enterotoxin that reversibly
288 increase intestinal permeability) inhibitor ameliorates T1D manifestation in rat model (141). Microbial
289 translocation in pancreatic lymph nodes activates NOD2 and IL-17 production in pancreatic lymph
290 nodes and pancreas contributes to T1D development (142).

291 Increased intestinal permeability precede **EAE** onset which worsen during disease progression (143).
292 Intestinal hyper-permeability was associated with the increase of crypt depth and mucosa thickness in
293 jejunum and ileum, as well as with an overexpression of zonulin 1 (143) as observed for T1D (141,144).

294 Intestinal barrier defect and subsequent exposure to microbial products play an important role in the
295 pathology of **SLE** (145,146). sCD14, lysozyme and CXCL16 are markers of antimicrobial response
296 found increased in SLE subject attesting of a defect of intestinal barrier (147).

297 **5.3 Intestinal Inflammation**

298 **T1D** is associated with increased intestinal myeloperoxidase activity and goblet cell (producing mucus)
299 density, supporting the idea that early intestinal inflammation might lead to intestinal
300 hyperpermeability (148,149). Many studies suggest that increase of Th17 cells is involved in the
301 pathogenesis of auto-immune diabetes. More IL-17 secreting cells were detected in recent-onset T1D
302 and IL-17 seems to promote inflammatory response to β -cells (150,151). Th17 are increased in
303 peripheral blood of children with T1D (151,152). *In vitro* IL-17 potentiate inflammatory and
304 proapoptotic responses on human islets cells (151). Anti-IL-17 treatment reduced islet T cell infiltrates
305 and GAD65 autoantibodies in NOD mice (153). Neutrophil extra cellular traps (NET) might contribute
306 to the generation of AD by exposing autoantigen (154). The role of NET has been particularly studied
307 in T1D. Indeed, degradation of NETs in the gut prevented immune infiltration of pancreatic islet
308 preserving β -cell mass and systemic inflammation (155).

309 In a mouse model of **SLE** developing severe nephritis, $\alpha 4\beta 7$ expressing T cells were increased in Peyer
310 patches and pro-inflammatory cytokines (IL-17, IL-22, IFN α and β) were much more expressed in
311 distal ileum (156). Furthermore, intestinal monocytes/macrophages of SLE patients have an altered
312 expression of type1 interferon stimulated genes, HLA-DR and Fc γ receptors (157,158). Monocytes
313 isolated from plasma of SLE patients release higher pro-inflammatory cytokines in response to LPS
314 compared to healthy patients (159). More generally, higher production of pro-inflammatory cytokines
315 by monocytes/macrophages has been described in SLE patients (for review (160))

316 In **MS**, elevated Th1 and Th17 pro-inflammatory responses were observed in *lamina propria*, Peyer's
317 patches and mesenteric lymph nodes (143). GF EAE animals produce lower levels of IFN γ and IL-17
318 in intestine associated with higher number of Treg cells (104). GF animals monocolonized with
319 segmented filamentous bacteria, known to induce IL-17 (161,162), develop EAE showing that
320 microbiota can affect neurologic inflammation by induction of Th17 in intestinal *lamina propria* that
321 recirculate to the brain causing inflammation (106). Autoreactive T cells could migrate in different
322 organs, from gut to brain in the case of MS, to liver in the case of auto-immune cholestatic liver disease
323 (163,164) or to kidney in the case of SLE (165). Interestingly, not only T cells seem to be involved in
324 MS but also circulating ILC. Indeed, higher number of ILC has been observed in MS patients (166).

325 **6 Conclusion**

326 As a conclusion, compiling evidence highlights the importance of both intestinal barrier defect and
327 stress in AD. Stress is well known to have long lasting deleterious consequences on intestinal barrier.
328 A transversal research on AD and intestinal barrier function would be of great interest and would bring
329 new understanding in the pathophysiology of AD. Identifying intestinal barrier as an actor of AD could
330 bring new possibilities for therapeutic targets and especially preventing strategies toward the spreading
331 epidemic of AD. Therapeutic strategies suggest that probiotics and FMT treatment might improve AD
332 symptom but preventive strategies in at risk population still need to be explore.

333 **7 Conflict of Interest**

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

336 **8 Author Contributions**

337 Hanna Ilchmann-Diounou and Sandrine Ménard reviewed the literature, wrote and corrected the
338 manuscript.

339 **9 References**

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CHAPTER IV

THE CONCEPT OF DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE (DOHAD)

It becomes clear that, the early life period is crucial for the development and maturation of metabolism, immune system, intestinal barrier and a life-long beneficial host-microbiota interaction. Early life adverse events, as for example infections, nutritional and psychological stress, poverty, family instability and abuse, can have not only immediate consequences on the host physiology but also long lasting consequences. Early life events have been identified as a powerful trigger in the development of various chronic diseases at adulthood (Gluckman, 2008). The long lasting effects of early life adverse events are understandable as perinatal period is a crucial and critical window where intestinal barrier, immune system and microbiota mature and establish an appropriate complex relationship leading to homeostasis (Stockinger et al., 2011).

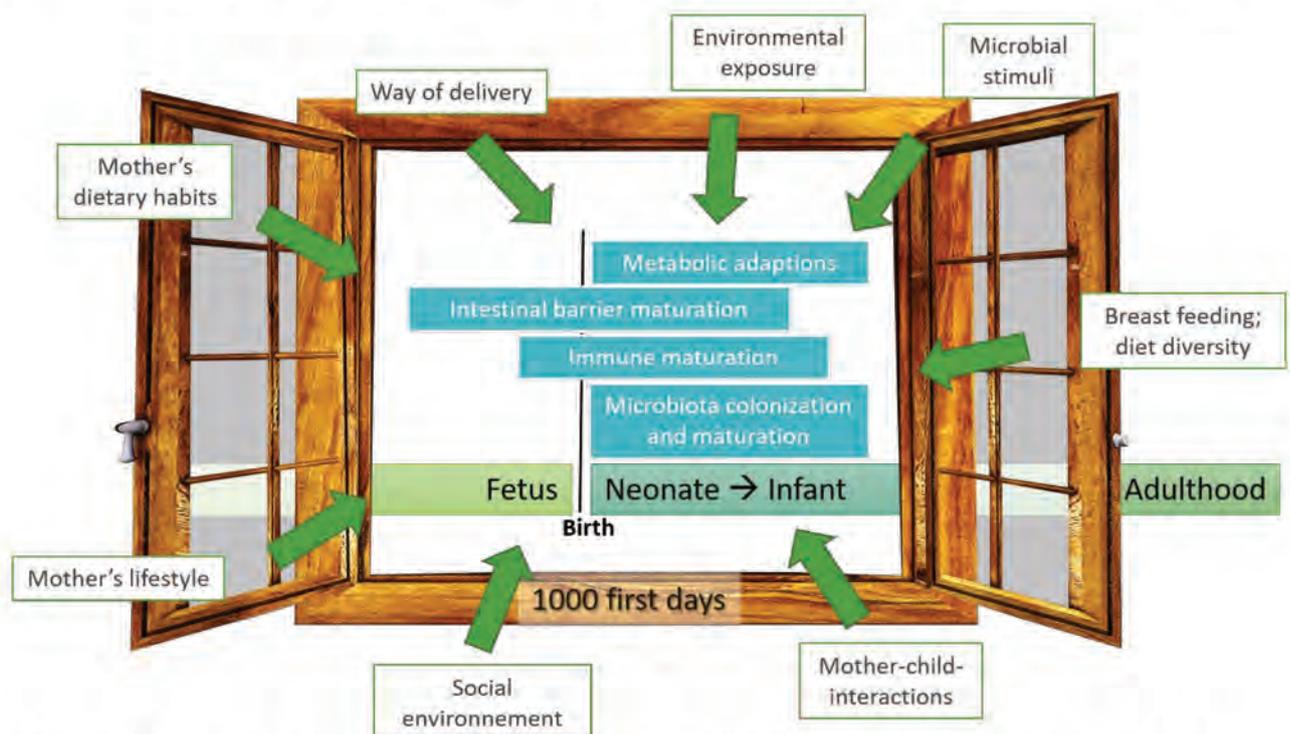


Figure 20 The neonatal window of opportunity. During the 1000 first days of a human life (from conception to the second birthday) various maturation processes take place. There are metabolic adaptations, intestinal barrier maturation, immune maturation and colonization by microbiota. These processes can be influenced and disturbed by several external factors.

The neonatal period is also important for metabolic adaptation. During the first months, the neonate is receiving a high-fat diet (maternal milk), which progressively switches into high-carbohydrate diet. These changes are demanding metabolic adaptations of glucose and lipid metabolism (Henning, 1981). Endocrine pancreas is also maturing in the neonatal time window,

β -cell mass is expanding, especially after weaning (Bonner-Weir et al., 2016). During the time of physiologic maturation, the organism is particularly sensitive to environmental influences (Figure 20).

1. Allostatic load

Over the past three decades, a body of epidemiologic evidences has shown that early-life conditions influence patterns of growth, body composition, and later risk of non-communicable diseases at adulthood. Among them there are type 2 diabetes (T2D), dyslipidemia, high blood pressure (for review (Gluckman, 2008; Weaver, 2009)) but also cancers (Kelly-Irving et al., 2013), autoimmune diseases like type 1 diabetes (T1D) (Virk et al., 2015) or cognitive and behavioral disorders (Banqueri et al., 2016).

Life course epidemiology proposes the concept of embodiment which is “how we, like any living organism, literally incorporate, biologically, the world in which we live, including our societal and ecological circumstances” (Krieger, 2005). Early life insults are incorporated, the allostatic load – which “is the cost of chronic exposure to fluctuating or heightened neural or neuroendocrine responses resulting from repeated or chronic environmental challenge that an individual reacts to as being particularly stressful” (McEwen and Stellar, 1993) - is increasing (Figure 21).

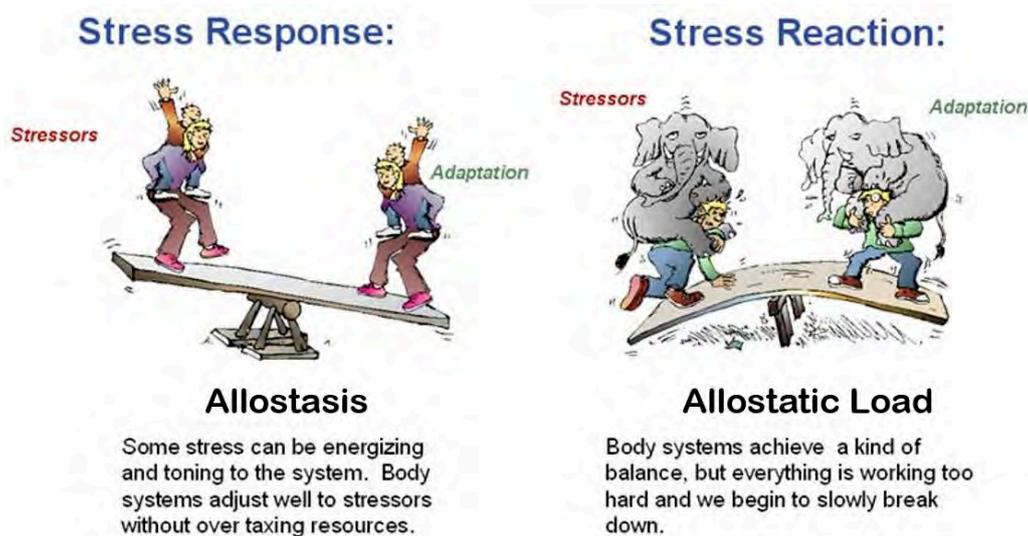


Figure 21 Allostasis and allostatic load. Suitable adaptive response of the organism to a stressor leads to allostasis. In case of increased stressors and increased adaptive response without relieve allostatic load is increasing (Seaway, n.d.).

2. Evidence for the DOHaD hypothesis

Barker proposed in the late 80s the concept of developmental origins of health and disease (DOHaD) (Barker et al., 1989) in response to observations he made in the Hertfordshire cohort. Indeed he found that low birth weight was associated with impaired glucose tolerance

in adulthood (Hales et al., 1991). Since, DOHaD has been extended from fetal period to a period running from meiosis and gametogenesis to adolescence, with special emphasis on the first 1000 days of life. Of interest is the health status in adulthood and even of those of the offspring's.

Studies about the consequences of the Dutch famine during World War II showed that adults, exposed to the famine in the second or third trimester *in utero* had lower glucose tolerance (Ravelli et al., 1998). Data on the Chinese famine in 1958 showed that adults exposed *in utero* had higher risk for T2D (Wang et al., 2016). There has also been established a link between intrauterine growth restriction and later dyslipidemia (Barker et al., 1993). Animal models allowed to confirm the observations from epidemiological studies. Uterine ligation is a model for idiopathic *in utero* growth restriction and leads to dyslipidemia and T2D (Lane et al., 2001; Simmons et al., 2001).

Even psychological diseases and IQ have been linked to early environment. Infants from mother with high cortisol levels during pregnancy had lower IQ than their siblings (LeWinn et al., 2009). In the cohorts of Chinese and Dutch famines, a more than doubled risk for developing schizophrenia has been observed for the exposed group (Hoek et al., 1996; St Clair et al., 2005). However, Serpeloni et al. reported that children, whose mother was exposed in prenatal and postnatal period to intimate partner violence, had less psychological disorders, as for example depression or anxiety, than children, which were only exposed in prenatal period to this stressor. They found epigenetic modifications on glucocorticoid receptor (*NR3C1*) and its repressor *FKBP51* (*FKBP5*) and differentially methylated regions on DNA, which has previously been associated with increased resilience (Serpeloni et al., 2019). However, these modifications might have beneficial effects only in the special environment where they are living (high violence). Indeed, the genes implicated are responsible for social and emotional behavior (oxytocin). The authors argue, that decreased sensibility to violence is protective in this specific context but may be disadvantageous in a more societally accepted subcultures (Serpeloni et al., 2019).

Modifications of immune system by early life environment have also been described. In human, O'Conner et al found that maternal prenatal anxiety predicts lower adaptive immune response after hepatitis B vaccination (O'Connor et al., 2013). Prenatal exposure to xenobiotic, as for example tobacco, increased asthma risk in infants (Hylkema and Blacquiere, 2009). In an animal model, perinatal exposure to bisphenol A led to altered immune response in adult

mice, namely dampened intestinal and increased systemic immune response (Malaisé et al., 2018, 2017).

3. Mechanisms

Mechanistic studies in the DOHaD domain are still sparse but underlying mechanisms, which has been proposed, are epigenetic modification of genome via DNA methylation and histone acetylation. The phenomenon of catch-up growth has been associated with endoplasmatic reticulum stress, which could trigger later deleterious metabolic outcomes. Metabolic adaption in response to nutritional stress in utero has been another proposition. In infants small for gestational age and in rodents with *in utero* growth restriction decreased lipid oxidation has been observed (Hoffman et al., 2017). A potent tool seems to be the allostatic load, which is measured by diverse biomarkers (Casavant et al., 2019; Thayer et al., 2016)

**II. PART
OBJECTIVES AND METHODOLOGY**



In the previous chapter, I presented that early life adverse events, as for example infections, nutritional and psychological stress (poverty, family instability and abuse) can have not only immediate consequences on the host physiology but also long lasting consequences. Early life events have been identified as a powerful trigger in the development of various chronic diseases at adulthood (Gluckman, 2008). During my PhD, we were particularly interested in neonatal social/psychological stress and its long lasting consequences on health and diseases.

Neonatal maternal separation (MS) is a model widely used in rodents as a paradigm of adverse events occurring early in life. Different types of maternal separation are described in the literature. Some separate dam from all or half of the litter, pups can be left alone or with the rest of the litter. MS can be applied once (acute stress) or can be repeated (chronic stress). Also the duration of separation vary in the different protocols (15 min to 24h). Besides, the time of exposition in the early life can be different.

In our laboratory maternal separation (MS) is performed daily for three consecutive hours (from 9 am to 12 pm). Pups are separated from their dam and the rest of the litter and kept at controlled temperature (27 ± 1 °C). MS is repeated for 10 working days, weekend excluded, between post-natal-day 2 (PND2) and PND15. Control pups are left undisturbed with their dam (**Figure 22**) (Barreau et al., 2004; Riba et al., 2018, 2017).

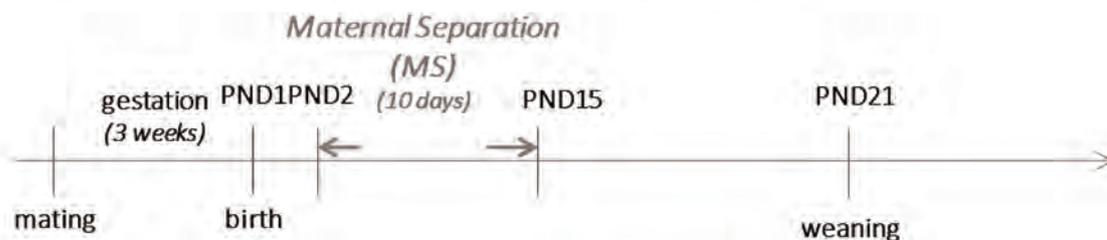


Figure 22 Neonatal maternal separation protocol. Pups are separated from the dam and the rest of the litter for three hours per day during ten days from post-natal day 2 to post-natal day 15 (weekend excluded). Animals are maintained in controlled temperatures and humidity. Control group is left undisturbed with the dam.

The model of maternal separation induced a large variety of outcomes. Indeed, there are studies interested in behavioral outcomes of early life stress (Gracia-Rubio et al., 2016). MS serves also as a model in the research on brain activity (Nishi et al., 2013) and depression (Vetulani, 2013). The model is also used to study the gut-brain axis (O'Mahony et al., 2011).

We are interested in this model due to its effects on intestinal homeostasis. Indeed, MS is responsible for long lasting alterations of intestinal homeostasis on adult male and female rodents offspring (Barreau et al., 2004; Riba et al., 2018, 2017).

Recent data from the host laboratory, who have been working with this model for some years, showed that MS induced in 50 days old (PND50) male mice an increase in intestinal permeability and visceral hypersensitivity. MS led to low-grade inflammation and microbiota dysbiosis. It also exacerbated immune response toward microbiota. Indeed, MS increased humoral response toward a commensal *E. coli* isolated from feces of the mouse strain used (Riba et al., 2018). MS induces already at PND50 a sexual dimorphic phenotype. In female 50-days old mice, MS leads to visceral hypersensitivity, decreased lysozyme expression, blooming of *E. coli*, humoral immune response against microbiota and intestinal low-grade inflammation (Riba et al., 2017).

We carried out separate studies for male and female mice, since sexual dimorphism was observed from PND50 on. We analyzed the long-term effects of MS on male and female mice separately.

The impaired intestinal barrier functions and abnormal immune responses toward microbiota observed in male mice by the MS model at PND50 have also been observed in metabolic disorders. For example, obesity and/or T2D have been associated with increased intestinal permeability in mice (Brun et al., 2007; Cani et al., 2008), IgG response against specific *E.coli* (Mohammed et al., 2012), dysbiosis (Turnbaugh et al., 2006) and low-grade systemic inflammation (Osborn and Olefsky, 2012). However, in PND50 male mice, which underwent maternal separation, no metabolic disorder were observed.

As metabolic disorders occur in aging individuals, we wondered if MS-induced low-grade inflammation leads to metabolic disorders in aging male mice (PND350) under normal diet and if we could identify MS as a risk factor in metabolic disorders development.

In our first study, we focused on the characterization of immunometabolism in aging male mice, which underwent MS in neonatal period. We aimed to analyze the long term

consequences of neonatal maternal separation on mice' metabolism, as well as on intestinal barrier functions, immune responses toward microbiota, and low-grade inflammation.

My work aims to provide experimental data to support a link between early life stress and development of metabolic disorders with aging. This project will highlight the neonatal period as a critical window for homeostasis establishment and early life adverse events like stress as a risk factor triggering metabolic disorders.

In a second time, we aimed to assess the role of microbiota dysbiosis in the development of metabolic disorders in the MS model in male mice. We wondered if microbiota dysbiosis, observed in the model of maternal separation, could trigger the observed immunometabolic phenotype in male mice. Fecal microbiota transfer is the current gold standard to prove causal relationship, so we carried out fecal microbiota transfer into germ-free male mice.

In a third project, we aimed to analyze long-term consequences of MS in female mice on allostatic load, namely intestinal barrier function, neuroendocrine functions and metabolism. Additionally, we were interested in immune response and wondered if MS could lead to autoimmune disorder in aging female mice. As previously discussed in the review article, there seems to be a link between stressful environment, intestinal barrier dysfunction and autoimmune disorders.

In a last part of this PhD project, we were interested in a methodological question regarding the various methods used to measure intestinal permeability and their relevance. We set up a project to compare *ex vivo* and *in vivo* measurements of intestinal permeability in a model of autoimmune type 1 diabetes. Our aim is to initiate a discussion around the definitions of intestinal permeability and systemic exposure in order to clarify the concept of intestinal permeability.

III. PART RESULTS



FIRST RESULT:
**EARLY LIFE STRESS INDUCES TYPE 2 DIABETES-LIKE FEATURES IN
AGING MICE**

Ilchmann-Diounou, Hanna; Olier, Maiwenn; Lencina, Corinne; Riba, Ambre; Barretto,
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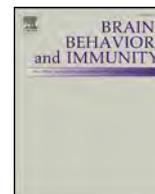
Brain, Behavior, and Immunity; August 2019

The first part of my work has the objective to describe long-lasting effects of neonatal maternal separation (MS) on immune-metabolism in male mice.

Previous data from our laboratory from Riba *et al.* showed that MS induced in 50 days old (PND50) male mice an increase in intestinal permeability and visceral hypersensitivity. MS led to low-grade inflammation and microbiota dysbiosis. It also exacerbated immune response toward microbiota. Indeed, MS increased humoral response toward a commensal *E. coli* isolated from feces of the mouse strain used (Riba et al., 2018).

These features are also described in metabolic disorders like diabetes. However, at PND50 no metabolic disorder has been observed. A major risk factor for metabolic disorders is aging. Therefore, we wondered, if mice, which underwent neonatal maternal separation would develop metabolic disorder with aging. This hypothesis was additionally strengthened by epidemiological studies, which showed an association between early life adverse events and metabolic disorder, especially type 2 diabetes (Huang et al., 2015).

This work has been published as an original article online on April 11, 2019, in print in August 2019 in the Journal “Brain, Behavior, and Immunity” and I signed as the first author.



Early life stress induces type 2 diabetes-like features in ageing mice

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ARTICLE INFO

Keywords:

Intestinal barrier
Microbiota dysbiosis
DOHaD
Non-communicable diseases

ABSTRACT

Early life stress is known to impair intestinal barrier through induction of intestinal hyperpermeability, low-grade inflammation and microbiota dysbiosis in young adult rodents. Interestingly, those features are also observed in metabolic disorders (obesity and type 2 diabetes) that appear with ageing. Based on the concept of Developmental Origins of Health and Diseases, our study aimed to investigate whether early life stress can trigger metabolic disorders in ageing mice.

Maternal separation (MS) is a well-established model of early life stress in rodent. In this study, MS increased fasted blood glycemia, induced glucose intolerance and decreased insulin sensitivity in post-natal day 350 wild type C3H/HeN male mice fed a standard diet without affecting body weight. MS also triggered fecal dysbiosis favoring pathobionts and significantly decreased IL-17 and IL-22 secretion in response to anti-CD3/CD28 stimulation in small intestine *lamina propria*. Finally, IL-17 secretion in response to anti-CD3/CD28 stimulation was also diminished at systemic level (spleen).

For the first time, we demonstrate that early life stress is a risk factor for metabolic disorders development in ageing wild type mice under normal diet.

1. Introduction

During the last century, the incidence of non-communicable diseases, including metabolic disorders, is expanding in western countries (Bach, 2002). The causes for this drastic increase are debated. The concept of Developmental Origins of Health and Disease (DOHaD) highlights the importance of early life period and raises the hypothesis that chronic diseases could find their origins in perinatal environment (Barker et al., 1989; Gluckman et al., 2016). In mice and humans, early life is important for the development of the immune system, metabolic switch, microbiota colonization (Tamburini et al., 2016) and the development of life-long beneficial host-microbe homeostasis (Hornef and Fulde, 2014). Adverse events can disturb these mechanisms of adaptation. Several observational epidemiological studies have shown an association between adverse childhood experiences and metabolic diseases in later life (Huang et al., 2015). This study aims to provide experimental data to support a link between early life stress and

development of metabolic disorders with ageing.

Metabolic disorders, such as obesity and type 2 diabetes are associated with modification of intestinal barrier, microbiota dysbiosis and low grade inflammation (Brun et al., 2007; Cani et al., 2008; Osborn and Olefsky, 2012; Turnbaugh et al., 2006). In mice, several models such as diet induced obesity (high-fat or western diets) or genetic models (*ob/ob* and *db/db*, respectively deficient for leptin and leptin receptor) are used to investigate obesity associated with hyperglycemia. In those models, a defect of intestinal barrier as well as low-grade inflammation were observed, even before the onset of obesity and hyperglycemia (Araújo et al., 2017; Brun et al., 2007). Neonatal maternal separation (MS) is a stress model widely used in rodents as a paradigm of early life adverse events. We previously observed that, in male mice, MS triggers long lasting alterations of intestinal homeostasis in young adult offspring (post-natal-day (PND) 50) including a defect of intestinal barrier, microbiota dysbiosis and low-grade inflammation (Riba et al., 2018). With ageing, intestinal permeability and low-grade

Abbreviations: DOHaD, Developmental Origin of Health and Diseases; FSS, Fluorescein Sodium Salt; HRP, Horse Radish Peroxidase; IBS, Irritable Bowel Syndrome; MS, Maternal Separation; OTU, Operational Taxonomic Unit; PND, Post-Natal Day; siLP, small intestine *Lamina Propria*; TCR, T cell Receptor

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<https://doi.org/10.1016/j.bbi.2019.04.025>

Received 5 November 2018; Received in revised form 22 February 2019; Accepted 10 April 2019

Available online 11 April 2019

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inflammation are increasing in mice (Thevaranjan et al., 2017) and human (Man et al., 2015). This might explain why chronic metabolic diseases appear in the ageing population and suggest a potential role of intestinal barrier defect itself in triggering and/or maintaining metabolic disorders.

Here, we aimed at investigating in wild-type male mice the long-term effects of neonatal MS on metabolism, intestinal barrier function, as well as on microbiota composition, immune response toward microbiota, and low-grade inflammation in ageing mice fed a regular diet.

2. Material and methods

2.1. Mouse model

All experimental protocols were approved by local Animal Care Use Committee (Comité d'Ethique de Pharmacologie-Toxicologie de Toulouse – Midi-Pyrénées, France) registered as N°86 at the Ministry of Research and Higher Education (N° 0029/SMVT), and conducted in accordance with the European directive 2010/63/UE. Mice were kept at a constant temperature ($22 \pm 1^\circ\text{C}$) and maintained on a 12:12 h light/dark cycle (lights on 7 h30 am) on Specific and Opportunistic Pathogen Free (SOPF) conditions. Normal diet (Harlan2018, Envigo, Gannat, France) and water were available *ad libitum*.

2.1.1. Maternal separation protocol

Nulliparous female C3H/HeN mice (Janvier Labs, Le Genest St Isle, France) were used. Maternal separation (MS) was performed as previously described (Riba et al., 2018). Briefly, pups were separated from their dam and the rest of the litter 3 hours per day. MS was repeated for 10 working days, week-end excluded, between post-natal-day 2 (PND2) and PND15. Control pups were left with their dam. At weaning (PND21), litters were mixed within the same group and housed by 5 mice per cage; only males were kept for this study as MS induced on PND50 male mice a defect of intestinal barrier, microbiota dysbiosis and low-grade inflammation (Riba et al., 2018), features also observed in metabolic disorders. Four independent batches of experiments were realized. Experiments were performed at PND350.

2.1.2. Oral glucose (OGTT) and intraperitoneal insulin tolerance test (ITT)

OGTT and ITT were performed in mice 6 h-fasted during day light. For OGTT, mice were gavaged with 2 mg glucose per g of bodyweight. Blood glucose levels were monitored from the tip of the tail vein with a glucose meter (Johnson & Johnson, Issy-les-Moulineaux, France) at –30, 0 (glucose gavage), 15, 30, 60, 90 and 120 min.

During ITT mice were injected with 0.75 mU insulin (NovoRapid, Novo Nordisk, Chartres, France) per g of bodyweight. Blood glucose levels were measured up to 30 min after injection.

For fasted blood glucose, mice were fasted 16-h overnight.

For plasma insulin, blood samples were harvested in fasted state (6 h) and 15 min after glucose stimulation *per os* (2 mg glucose per g of bodyweight). Insulin was measured with commercial ELISA kit (Merck Millipore, Saint Quentin en Yvelines, France).

2.2. Fecal microbiota composition analysis

Total microbial genomic DNA was obtained from stool samples using the ZR Fecal DNA Miniprep™ (Zymo Research, Ozyme SAS, Montigny-le-Bretonneux, France) and DNA quantity was determined using a TECAN Fluorometer (Qubit® dsDNA HS Assay Kit, Molecular Probes, ThermoFisher Scientific, Montigny le Bretonneux). The microbial 16S rRNA gene was amplified during the first PCR step with adapter fusion primers targeting the V3 to V4 regions. Sequence reads were quality controlled and treated first with the FROGS pipeline (Find Rapidly OTU with Galaxy Solution) (Escudé et al., 2018) to obtain OTUs and their respective taxonomic assignment thanks to Galaxy instance (<http://sigenae-worbench.toulouse.inra.fr>). Rarefaction curves,

richness and diversity indexes of bacterial community, as well as ordinations were computed using the Phyloseq package (v 1.19.1) in RStudio software (McMurdie and Holmes, 2012; McMurdie and Holmes, 2013; R Development Core Team, 2011). Differences in structure between groups were determined using Adonis (permuted p-value was obtained by performing 9999 permutations). LDA effect size was performed between two groups and plotted using LefSe (Segata et al., 2011). For further univariate differential abundances analysis, closely-related taxa were agglomerated at the species rank, reducing the taxon list to 73. A negative binomial fit model for count data was run on all groups using the DESeq2 package (v 1.14.1) (Love et al., 2014; McMurdie and Holmes, 2014). Tests were corrected for multiple inferences using the Benjamini-Hochberg method to control the false discovery rate (Hochberg and Benjamini, 1990). Complete methods and accession numbers are available in the [Supplementary methods](#).

2.3. Intestinal permeability in Ussing chambers

Intestinal permeability was assessed as previously described (Riba et al., 2018). Briefly, jejunal and colonic fragments were mounted in Ussing chambers (Physiologic Instruments, San Diego, CA, USA). Tissues were bathed 2 h with oxygenated thermostated Krebs's solution (Sigma, Saint Quentin Fallavier, France). Fluorescein Sodium Salt 40 µg/ml (FSS 376 Da; Sigma) and Horse Radish Peroxidase 0.4 mg/ml (HRP 4 kDa; Sigma) were respectively added to mucosal compartment as para- and trans-cellular markers of intestinal permeability.

Epithelial permeability to total HRP was determined by ELISA. Briefly, 96-wells black plates (Greiner, Les Ulis, France) were coated with 10 µg/ml mouse polyclonal to HRP (Abcam, Paris, France), blocked with PBS-1% bovine serum albumin (BSA), incubated with serosal compartments of Ussing chamber, detected with 10 µg/ml Rabbit polyclonal anti HRP biotin (Abcam) and revealed with FITC-conjugated streptavidin (BD, Paris, France). Fluorescence intensity was measured 485 nm/525 nm using an automatic Infinite M200 microplate reader (Tecan, Männedorf, Switzerland). Epithelial permeability to FSS was determined by measuring the fluorescence intensity (FI) 485 nm/525 nm using an automatic Infinite M200 microplate reader. Permeability was calculated as the ratio of flux/concentration, and expressed as cm/second.

2.4. Immune cells isolation

Splenocytes were isolated through a 70-µm nylon mesh and suspended in PBS 1%-KO SR serum (Gibco, ThermoFisher Scientific).

Isolated cells from Small Intestines (si) lamina propria (siLP) were obtained as follow: si were washed in cold PBS, incubated in PBS 3 mM EDTA (Sigma), washed in warm PBS, digested with 100 U/mL of collagenase (Sigma) in DMEM 20% FCS and finally purified on Percoll (Sigma).

2.4.1. Fluorescence-Activated cell sorter analysis

Isolated cells from spleen and siLP were stained as follow (all antibodies are listed in [Table 1](#)). Activated T-cells: CD4 (BD), CD44 (BD), CD62L (BD); Regulatory T-cells: CD4 (BD), CD25 (BD), Foxp3 (ebioscience, ThermoFisher Scientific); ILC3: CD127 (BD), RORγt (BD). Th17/22 CD3 (BD), RORγt (BD), IL-17 (BD). MACSQuant® Analyzers (Miltenyi Biotec SA, Paris, France) and VenturiOne® (AppliedCytometry, Sheffield, Great Britain) software were respectively used for data collection and analysis.

2.4.2. Primary cell culture

Isolated cells from spleen and siLP were seeded at 2×10^6 cells per well in the presence or absence of a) 100 ng/mL Lipopolysaccharide (LPS; Sigma) or b) 5 µg/mL hamster anti-mouse CD3 (BD) and hamster anti-mouse CD28 (BD) coated wells. Supernatants were collected after a) 24 h, b) 72 h.

Table 1
Antibodies.

Antibody	Company	Reference	Working concentration
Anti-CD3-FITC	BD	553062	dil 1/200
Anti-CD3	BD	555273	5 µg/mL
Anti-CD4-PE	BD	553730	dil 1/200
Anti-CD25-PE-Cy7	BD	552880	dil 1/100
Anti-CD28	BD	553295	5 µg/mL
Anti-CD44-APC-Cy7	BD	560568	dil 1/200
Anti-CD62L-PE-Cy7	BD	560516	dil 1/500
Anti-CD127-AF488	BD	561533	dil 1/50
Anti-FoxP3 PerCP-Cy5.5	eBioscience	45-5773-82	dil 1/100
Anti-RORγT-A647	BD	562682	dil 1/100
Anti-IL17-PE	BD	559502	dil 1/100
Anti-mouse-Ig	Southern Biotech	1010-01	5 µg/mL
Anti-mouse-IgG-HRP	Southern Biotech	1030-05	dil 1/8000
Anti-mouse-IgA	Sigma	M-1272	5 µg/mL
Anti-mouse-IgA-HRP	Sigma	A4789	1.5 µg/mL
Anti-HRP	Abcam	34961	10 µg/mL
Anti-HRP-biotin	Abcam	195239	1.6 µg/mL

2.5. Cytokines measurement

Cytokines were measured in supernatant of primary cell culture, or jejunal fragments suspended in RIPA buffer (0.5% deoxycholate, 0.1% SDS and 1% Igepal in TBS) containing complete anti protease cocktail (Sigma). Jejunal protein concentrations were measured using BCA up-tima kit (Interchim, Montlucon, France). IL-17, TNF α , IL-10, IL-22, TGF β and IFN γ in supernatant or lysate of jejunal fragments were assayed using commercial ELISA kits (R&D Systems, Lille, France).

2.6. Humoral response in feces and plasma

Plates were coated with 5 µg/ml of sheep anti-mouse IgA (Sigma) or goat anti-mouse IgG (SouthernBiotech, Cliniscience, Nanterre, France), incubated with plasma, detected with 1.5 µg/ml HRP-conjugated goat anti-mouse IgA (Sigma) or goat anti-mouse IgG (SouthernBiotech), HRP was revealed using TMB and the reaction was stopped with H₂SO₄ before reading at 450 nm using automatic Infinite M200 microplate reader.

Immunoglobulin specificity against commensal *E. coli*

Maxisorp 96-wells plates were coated with 5 µg/ml of protein from C3H/HeN isolated *E. coli* lysate (being used as representative bacteria of the intestinal microbiota), incubated with plasma (10 µg/mL IgG), and revealed as above-mentioned. Results were expressed as arbitrary units (AU) per 10 µg/mL of IgG, in comparison with a standardized immune serum. The *E. coli* lysate was prepared as previously described (Riba et al., 2018).

2.7. Measurements in plasma

ELISA kits were used to monitor corticosterone (Immunodiagnostic Systems, Pouilly-en-Auxois, France), GIP, GLP-1 (Merck Millipore), Ghrelin (elabioscience, Clinisciences) and LPS-binding protein (LPB) (Abnova, Cliniscience) in plasma. Plasma-cholesterol, LDL, HDL, triglycerides, free fatty acids and calcium were analyzed by the Platform GenoToul Anexplo, Toulouse, France.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.04 (GraphPad Software, La Jolla, CA, USA). Results for single comparisons were displayed as box plots [min to max] and analyzed using Student's unpaired t-test or Mann-Whitney test after prior Shapiro-Wilk

Normality test and F-Test to compare variances. Results in text were described as MS mean \pm SD vs. Control mean \pm SD for normally distributed samples and as median, [25%-quartile; 75%-quartile] in other case. Multiple conditions in OGTT, ITT and cytokine measurements were displayed either as kinetics with SEM or box plots [min to max] and compared per family by Holm-Sidak posttest after a significant repeated measures (RM) two-way ANOVA. Differences were considered significant for $P < 0.05$.

3. Results

3.1. MS's effects on glucose tolerance and insulin sensitivity

At post-natal-day 350 (PND350), MS did neither affect body weight (Fig. 1A) or the perigonadal-adipose-tissue-weight (PGAT)-to-body-weight-ratio (data not shown) nor feed intake (Fig. 1B). However, MS mice had higher fasted blood glucose than the control mice (127.0 mg/dL \pm 23.5 vs. 101.1 mg/dL \pm 22.3, $t_{13} = 2.175$, $p < 0.05$, two tailed t-test, Fig. 1C). During oral glucose tolerance test (OGTT), blood glucose levels were higher in MS mice than in control (at 15 min: 270 mg/dL \pm 57, vs. 227 mg/dL \pm 57, $p < 0.0001$; at 30 min: 211 mg/dL \pm 47 vs. 178 mg/dL \pm 30, $F_{1,49} = 6.668$, RM-two-way ANOVA followed by Holm-Sidak's post test, $p < 0.01$, Fig. 1D). The Area Under the Curve (AUC) during OGTT for MS mice were higher (21353 mg/dL/2h, [20460; 24068], vs. 20022 mg/dL/2h, [18376; 21866], $t_{49} = 2.463$, $p < 0.05$, two tailed t-test, Fig. 1E). MS did affect neither fasted nor glucose-stimulated insulin secretion (Fig. 1F). Insulin tolerance test (ITT) suggests lower insulin sensitivity for MS mice. Slower decrease of blood glucose (at 15 min: 136.5 mg/dL \pm 27.6, vs. 105.1 mg/dL \pm 26.1, $p < 0.01$; at 30 min: 97.9 mg/dL \pm 24.5 vs. 69.7 mg/dL \pm 15.6, $F_{1,28} = 7.365$, RM-two-way ANOVA followed by Holm-Sidak's post test, $p < 0.01$, Fig. 1G), resulted in significantly higher Area Under the Curve (AUC) for MS mice (3997 mg/dL/30 min \pm 162.8 vs. 3306 mg/dL/30 min \pm 139.2, $t_{28} = 3.105$, two tailed t-test, $p = 0.0043$, Fig. 1H). In plasma, no difference in corticosterone (Fig. 2A), incretins (GIP, GLP-1) (Fig. 2B and C), cholesterol, HDL, LDL, triglycerides or free fatty acids (FFA) (Fig. 2D–H) levels was observed. However, MS significantly increased ghrelin in plasma (0.61 \pm 0.13, $n = 10$ vs. 0.50 \pm 0.05, $t_{18} = 2.496$, two tailed t-test, $p = 0.0279$, Fig. 2I) at PND350.

3.2. Effect of MS on fecal microbiota

Despite the bacterial community richness indicated by the α -diversity richness index showed no significant change between MS mice and control group (230 OTUs \pm 5.86, vs 244 \pm 8.56; $t_{14} = 1,409$, two-tailed t-test, $n = 9$) (Fig. 3A), β -diversity indices revealed alterations in the taxonomic bacterial community structure in MS compared to control mice. Indeed β -diversity determined using both unweighted and weighted Unifrac distances was altered in response to early life stress ($F_{1,14} = 3,775$; $p = 0.0002$ and $F_{1,14} = 2,71$; $p = 0.043$ respectively) (Fig. 3B). As compared to weighted Unifrac distance, use of the unweighted Unifrac distance revealed a more pronounced separation between mice groups, suggesting that abundant OTUs in both groups are phylogenetically close and that MS-induced alterations mainly affect OTUs with lower abundance. These findings were confirmed by OTUs prevalence visualization (Supplementary Fig. 1A) and by examination of differences at each taxonomic rank using LEfSe analysis (Fig. 3C, Supplementary Fig. 1B). No appreciable differences at the taxonomic level of phyla was indeed observed between MS mice and control group. However, community composition of MS mice was increased in both *Betaproteobacteria* and *Gammaproteobacteria* classes within the *Proteobacteria* phylum, as well increased in *Bacteroidaceae* and *Enterococcaceae* families within the *Bacteroidetes* and *Firmicutes* phyla respectively. Reduced abundance in *Rikenellaceae* and additional taxa mainly among *Lachnospiraceae* family was observed in MS mice as

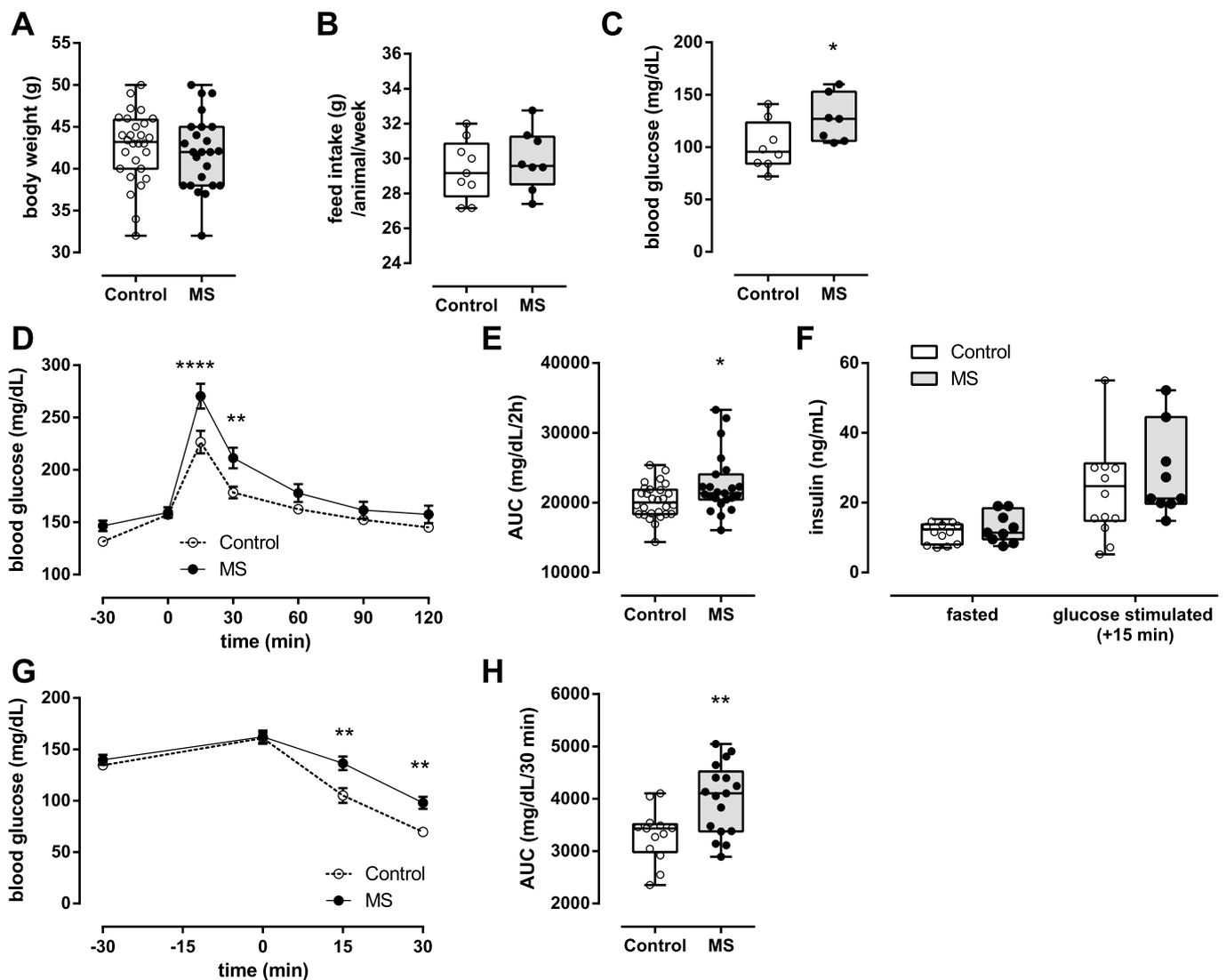


Fig. 1. MS induced oral glucose intolerance associated with a loss of insulin sensitivity at PND350. (A) Body weight (g), $n = 24\text{--}28$. (B) Feed intake (g)/animal/week, $n = 8\text{--}9$. (C) 16-h fasted blood glucose levels (mg/dL), $n = 7\text{--}8$, unpaired t-test, $*p < 0.05$. (D) Oral glucose tolerance test, after 6-h fasting, gavage with 2 mg glucose/g bodyweight at 0, blood glucose (mg/dL) from -30 min to 120 min, $n = 23\text{--}28$, RM-two-way-ANOVA, $**p < 0.01$, $****p < 0.0001$. (E) Area under the curve of blood glucose 0–120 min (mg/dL/2h), $n = 23\text{--}28$ Mann-Whitney test, $*p < 0.05$. (F) Plasma insulin (ng/mL) after 6-h fasting and 15 min after oral glucose stimulation (2 mg glucose/g bodyweight). (G) Insulin tolerance test, after 6-h fasting, intraperitoneal injection of 0.75 mU insulin/g bodyweight at 0, blood glucose (mg/dL) from -30 min to 30 min, $n = 13\text{--}17$, RM- two-way-ANOVA, $**p < 0.01$. (H) Area under the curve of blood glucose 0–30 min (mg/dL/30 min), $n = 13\text{--}17$, unpaired t-test, $**p < 0.01$.

compared to control mice (Supplementary Fig. 1B). Once agglomerated at the species rank, differential abundance analysis using DESeq size factors revealed that taxa significantly enriched in MS mice were pathobionts, i.e. *Bacteroides vulgatus*, *Proteus mirabilis*, *Enterococcus faecalis*, *Escherichia coli*, and unidentified species belonging to *Parasutterella* and *Bacteroides* genus, whereas one unclassified taxa belonging to commensal *Lachnospiraceae* (among 11 additional different unclassified *Clostridiales* members at the OTUs level) was significantly enriched in control mice (q -values < 0.05 ; Fig. 3D, Supplementary Fig. 1C, Table 2). In sum, alterations in structure and composition observed in fecal microbiota of MS mice at PND350 meet the definition of dysbiosis proposed notably by Peterson and Round (Peterson and Round, 2014), i.e. a disturbance in the microbiome structure that may consist in a loss of beneficial microorganisms, and/or expansion of pathobionts or harmful microorganisms.

3.3. Repercussions of MS on intestinal permeability

We then wondered if MS could have long lasting consequences on intestinal barrier. First, translocation of intestinal bacterial fragments was assessed indirectly by LPS Binding Protein (LBP) concentrations in plasma (Abad-Fernández et al., 2013), without modification in MS mice (Fig. 4A). Additionally, we addressed intestinal permeability *ex-vivo* in Ussing chambers in jejunum (Fig. 4B–D) and colon (data not shown). No difference for electrical resistance, para- (Fluorescein Sodium Salt, FSS) and trans-cellular permeability (Horse Radish Peroxidase, HRP) was observed between MS and control mice.

3.4. Effects of MS on intestinal immune system

Even though MS had no consequences on intestinal permeability, we wondered if humoral immune response was altered. Fecal IgG concentrations were decreased in MS mice compared to control mice ($0.1078 \mu\text{g}/\text{mg}$ fecal protein [0.0375; 0.1625] vs. $0.2018 \mu\text{g}/\text{mg}$ fecal

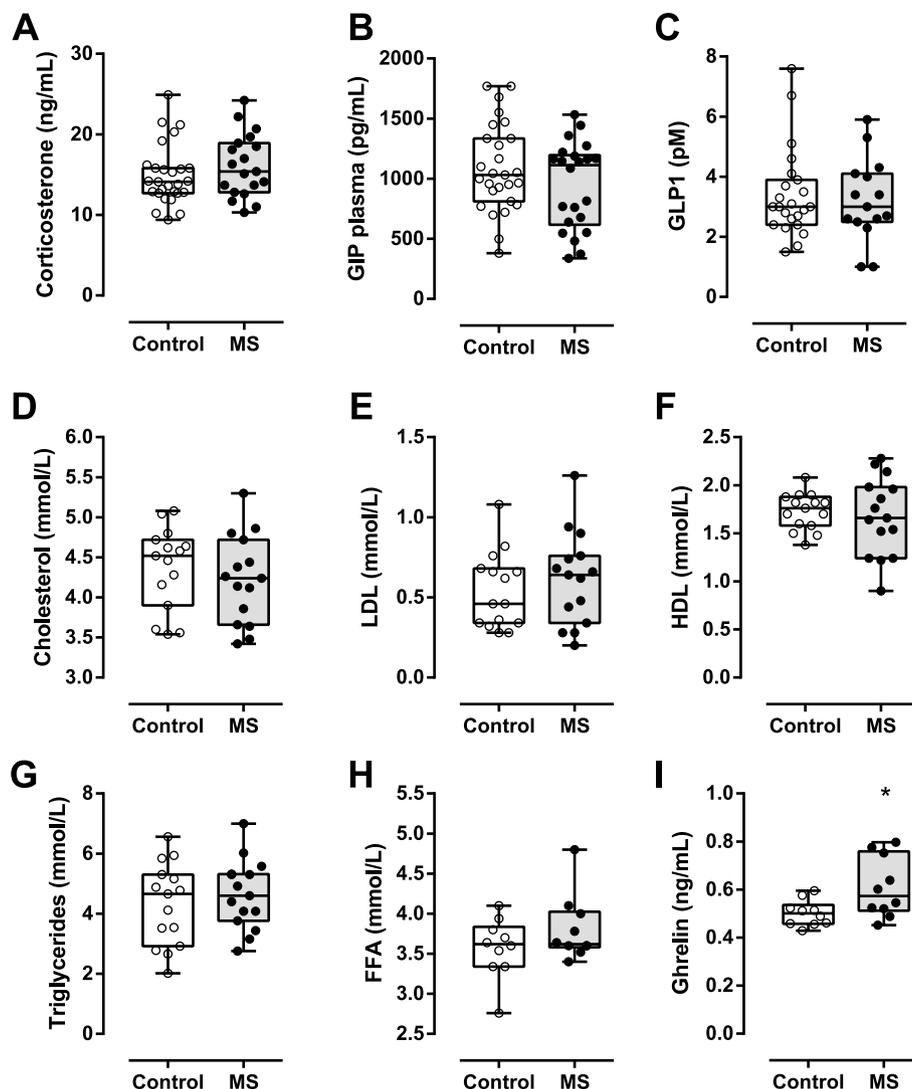


Fig. 2. Plasmatic measurements in MS and control mice at PND350. (A) Basal plasma corticosterone levels (ng/mL) at PND350, $n = 19$ –27. (B) Plasma GIP (pg/mL), $n = 22$ –27. (C) Plasma GLP1 (pM) in anti-DPP4 treated plasma, $n = 15$ –23. (D) Plasma cholesterol (mmol/L), $n = 15$. (E) Plasma LDL (mmol/L), $n = 15$. (F) Plasma HDL (mmol/L), $n = 15$. (G) Plasma triglycerides (mmol/L), $n = 15$. (H) Plasma free fatty (FFA) acids (mmol/L), $n = 10$. (I) Ghrelin (ng/mL), $n = 10$, unpaired t-test with Welch's Correction, $*p < 0.05$.

protein [0.1066; 0.3825], $t_{82} = 3.044$, two tailed t-test $p = 0.0006$, Fig. 5A). However, fecal IgA content was not different (Fig. 4B). Plasmatic IgG and IgA concentrations were similar between both groups (Fig. 5C and D) whereas anti-*E. coli* IgG representing humoral response toward commensal microbiota were significantly increased in plasma of MS mice (673 AU/10 μ g/ml IgG [362.7; 904.8], vs. 449.4 AU/10 μ g/ml IgG [274.6; 754.5], $t_{80} = 2.145$, two tailed t-test, $p = 0.0362$, Fig. 5E).

We analyzed cellular immune response in the siLP. Percentage of $Ror\gamma^+$ in $CD3^+$ was not affected by MS (Fig. 6A). Innate Lymphoid Cells 3 (ILC3: $CD127^+ ROR\gamma^+$) (Fig. 6B) and regulatory T cells (Treg: $CD25^+ Foxp3^+$ in $CD4^+$) (data not shown) were not altered in MS mice. Fluorescence intensity of IL-17 on Th17 cells ($CD3^+ ROR\gamma^+ IL-17^+$) was significantly decreased for MS mice suggesting lower IL-17 production (3428 IF IL-17 \pm 1567 vs. 7854 IF IL-17 \pm 2599, $t_5 = 2.836$, two tailed t-test, $p = 0.0364$, Fig. 6C). Furthermore, IL-17 secretion in response to TCR stimulation (anti-CD3/28) (Fig. 6D) and IL-17 in jejunal tissue (Fig. 6E) were decreased in MS mice (TCR stimulation: 2191.3 pg/ml \pm 1583 vs. 4753.8 pg/ml \pm 3635, $F_{1,21} = 4.610$, RM-two-way ANOVA followed by Holm-Sidak's post test, $p < 0.01$; jejunal tissue: 30.98 pg/mg \pm 19.1 vs. 49.36 pg/mg \pm 21.1, $t_{20} = 2.145$, two tailed t-test $p = 0.0444$). $IFN\gamma$ -secretion was not modified by MS (Fig. 6F). Nevertheless, IL-10 and IL-22-secretion in

response to TCR-stimulation was significantly decreased in isolated LP cells from MS mice as observed for IL-17 (IL-22: 2013.4 pg/ml \pm 800 vs. 4597.7 pg/ml \pm 1115, $F_{1,21} = 3.028$, RM two-way ANOVA followed by Holm-Sidak's post test, $p < 0.05$ Fig. 6G and H; IL-10: 709.8 pg/ml \pm 961 vs. 2045.1 pg/ml \pm 2056, $F_{1,15} = 2.969$, RM-two-way ANOVA followed by Holm-Sidak's post test, $p < 0.05$). Additionally, IL-10 concentrations were decreased in jejunum of MS mice (82.82 pg/mg \pm 45.8 vs. 138.7 pg/mg \pm 33.6, $t_{23} = 3.454$, two tailed t-test, $p = 0.0022$; Fig. 6I), whereas IL-22 were non-detectable and $TGF\beta$ similar in both groups (data not shown). $TGF\beta$ -secretion in response to TCR stimulation was not modified by MS (data not shown). Finally, $TNF\alpha$ -secretion in response to LPS-stimulation was slightly but significantly increased in cells from MS mice (32.25 pg/ml \pm 32.06 vs. 11.54 pg/ml \pm 13.02, $F_{1,21} = 2.996$, RM-two-way ANOVA followed by Holm-Sidak's post test, $p < 0.05$, Fig. 6J).

3.5. MS's effects on systemic immune response

We next assessed the systemic consequences of MS. Activated T cells ($CD4^+ CD44^{high} CD62L^{low}$) (Fig. 7A) and regulatory T cells ($CD4^+ CD25^+ foxp3^+$) (Fig. 7B) populations in spleen were not modified by MS. Regarding functionality, $IFN\gamma$ and IL-10-secretion in response to

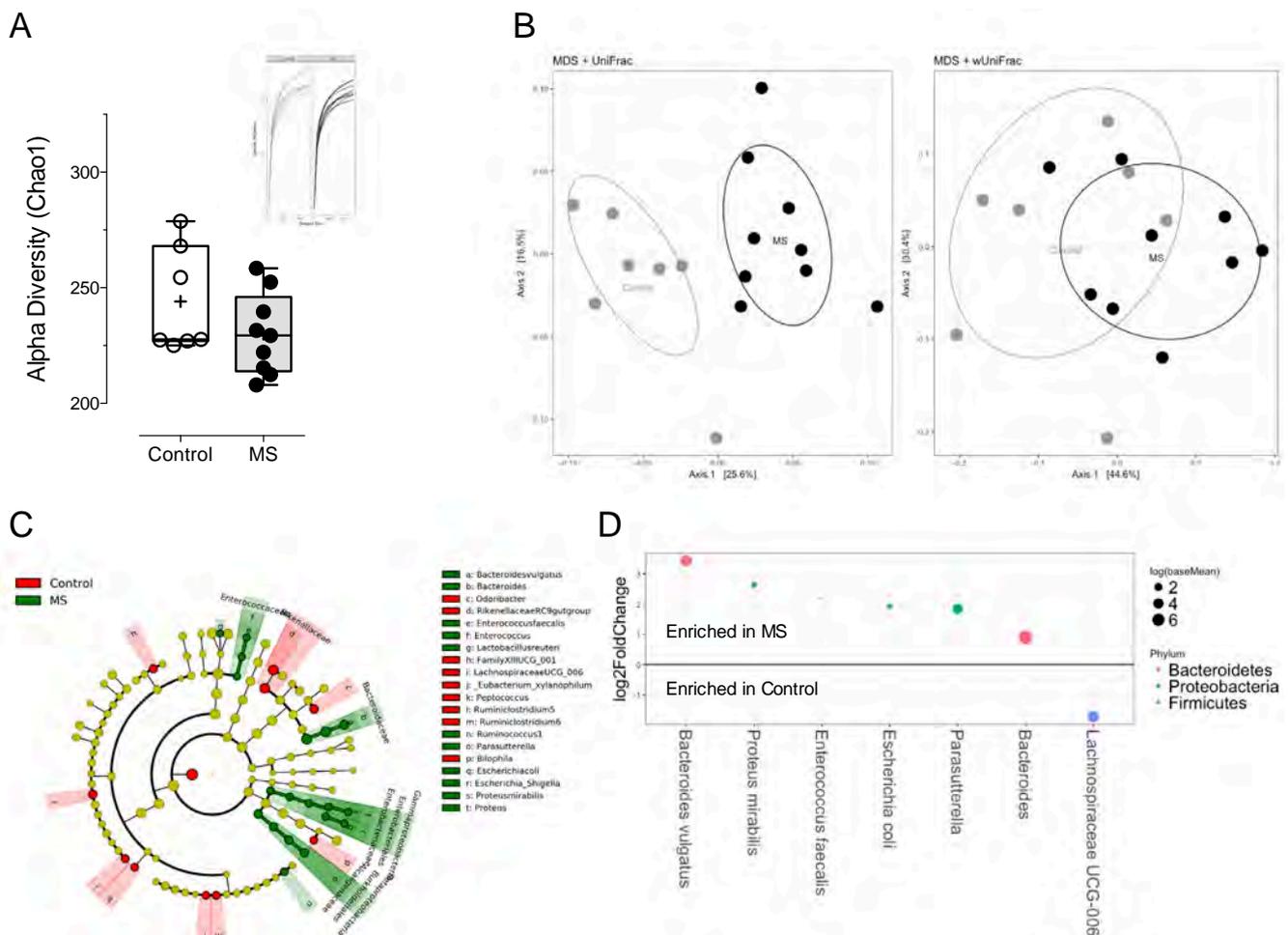


Fig. 3. MS induced fecal microbiota dysbiosis in favor of pathobionts. (A–E) 16S rRNA gene illumina Miseq sequences analysis, $n = 7–9$. (A) Chao-1 diversity index in male PND350 mice. (B) Unweighted and weighted Unifrac Multidimensional Scaling (MDS) plot representing structural changes in bacterial community composition in MS and control mice. (C) Circular cladogram generated from LEfSe analysis showing the most differentially abundant taxa enriched in microbiota from control mice (red) or MS mice (green). LDA scores > 3 and significance $\alpha < 0.05$ determined by Kruskal-Wallis test. (D) Classified differentially abundant taxa between MS and control mice. $\log_2(\text{MS}/\text{Control}) = \log_2(\text{MS}/\text{Control})$ is plotted on the Y-axis. Phylum is indicated using color codes. Features were considered significant if their adjusted post test p-value was < 0.05 . Key: a: *Bacteroides vulgatus*, b: *Bacteroides* spp., c: *Odoribacter* spp., d: Rikenellaceae RC9 gut group, e: *Enterococcus faecalis*, f: *Enterococcus* spp., g: *Lactococcus reuteri*, h: Family XIII UCG-006, j: *Eubacterium xylanophilum*, k: *Peptococcus* spp., l: Uncl. *Ruminiclostridium* 5, m: Uncl. *Ruminiclostridium* 6, n: Uncl. *Ruminococcus* 1, o: *Parasutterella* spp., p: *Bifidobacterium* spp., q: *Escherichia coli*, r: *Escherichia shigella* genus, s: *Proteus mirabilis*, t: *Proteus* spp. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

TCR-stimulation were not modified by MS (Fig. 7C and D). However, IL-17 secretion was diminished in response to TCR-stimulation ($4249.81 \text{ pg/ml} \pm 1902.5$ vs. $6590.09 \text{ pg/ml} \pm 4659.7$, $F_{1,33} = 3.348$, RM-two-way ANOVA followed by Holm-Sidak's post test, $p < 0.05$, Fig. 7E). TNF α -secretion in response to LPS-stimulation was slightly but significantly increased in splenocytes of MS mice ($243.81 \text{ pg/ml} \pm 116.7$, vs. $197.30 \text{ pg/ml} \pm 50.8$, $F_{1,46} = 4.293$, RM-two-way ANOVA followed by Holm-Sidak's post test, $p < 0.05$, Fig. 7F). Overall, MS-induced immunological perturbations followed the same tendency at systemic and intestinal level.

4. Discussion

This study shows, for the first time, that early life stress is a risk factor for glucose intolerance and loss of insulin sensitivity in ageing wild-type mice under normal diet. In this model, glucose intolerance was associated with microbiota dysbiosis, systemic response against microbiota and decrease of IL-17 and IL-22 response in the intestine.

We previously observed that MS triggered features of Irritable Bowel Syndrome (IBS) in young adult C3H/HeN male mice (PND50): visceral hypersensitivity, intestinal hyperpermeability, low-grade

inflammation, defect of Paneth cells and microbiota dysbiosis (Riba et al., 2018). As those features, apart of visceral hypersensitivity, are also observed in metabolic disorders, we wondered if MS could trigger metabolic disorders with ageing as metabolic disorders are ageing related diseases. This hypothesis was strengthened by epidemiological study showing that both, stress and IBS, are positively correlated with higher HbA1c (glycated hemoglobin) in patients suffering from type 2 diabetes (Badedi et al., 2016) indicating worse glycemic control. Furthermore, epidemiological studies showed that IBS is related to metabolic disorders independently of dietary patterns (Gulcan et al., 2009; Guo et al., 2014). At PND350, MS induces glucose intolerance associated with a loss of insulin sensitivity. Stress hormones (glucocorticoids) known to regulate metabolism (Schäcke et al., 2002) like corticosterone are not affected by MS at PND350 excluding a direct effect of corticosterone on the observed metabolic phenotype.

Scattering evidences suggesting consequences of MS on metabolism have already been published. MS alone did not induce glucose intolerance in 8-months-old Sprague-Dawley male rats, but diminished insulin receptor expression in muscle and serum IGF-1 levels (Ghosh et al., 2016). However, Sprague-Dawley rats submitted to MS combined with post-weaning social isolation developed glucose intolerance at

Table 2
Taxonomic affiliation of clusters agglomerated at the species rank affected by MS in early life.

Phylum	Class	Order	Family	Genus	Species	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	Bacteroides	2323,5	0,907	0,289	3,141	0,00168	0,02454
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	Bacteroides vulgatus	142,7	3,430	0,823	4,168	0,00003	0,00224
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospiraceae	UCG-006	139,9	-1,722	0,495	-3,480	0,00050	0,00915
Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	Enterococcus faecalis	1,5	2,193	0,749	2,929	0,00340	0,04137
Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Parasutterella	Parasutterella	42,2	1,839	0,493	3,732	0,00019	0,00693
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia-Shigella	Escherichia coli	1,9	1,916	0,684	2,804	0,00505	0,05270
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Proteus	Proteus mirabilis	2,1	2,639	0,731	3,610	0,00031	0,00745

PND180, involving increased corticosterone levels (Vargas et al., 2016) reinforcing the idea that long-lasting perturbations of metabolic homeostasis could enhance the risk for metabolic dysfunctions through higher vulnerability to additional risk factors (Ghosh et al., 2016). Finally, shorter MS protocol (5 days, 90 minutes per day) increased body weight as well as glucose and insulin response after arginine-stimulation in male Sprague-Dawley rats aged between PND105 and PND133 (Gehrand et al., 2016).

Interestingly, in our study, the MS-induced glucose intolerance and loss of insulin sensitivity were not associated with changes in body weight. This is of particular interest as in human, type 2 diabetes are not always associated with obesity (Carnethon, 2014; Zou et al., 2017).

Neither plasmatic markers mainly involved in metabolic disorders (FFA, triglycerides, cholesterol, HDL, LDL) nor incretins, like GLP-1 and GIP, were modified by MS at PND350. However, ghrelin, a satiety hormone, was significantly increased in MS mice compared to control without modification of food intake. In *ob/ob* mice loss of ghrelin production significantly raised insulin secretion restoring peripheral insulin sensitivity, thus improving glucose homeostasis (Sun et al., 2006). So, in our model increase of ghrelin might contribute to the glucose intolerance and the loss of insulin sensitivity.

MS consequences on metabolic disorders were associated with microbiota dysbiosis. Microbial signature at PND350 in response to MS displayed similitudes with signature previously observed in patients with type 2 diabetes, that was mainly characterized by a moderate degree of enrichment in *Bacteroidetes* (mainly explained by a bloom in *Bacteroides* genus) and *Betaproteobacteria*, associated with a decrease in *Clostridia* within *Firmicutes* phylum (Larsen et al., 2010; Leite et al., 2017; Pedersen et al., 2016). As previously observed in type 2 diabetic patients, using a metagenome-wide association study (Qin et al., 2012), microbiota dysbiosis in response to MS at PND350 was also mainly driven by pathobionts like *Bacteroides vulgatus*, *Enterococcus faecalis* as well as *Enterobacteriaceae* such as *Proteus mirabilis* and *Escherichia coli* (Leite et al., 2017; Cuív et al., 2017; Seo et al., 2015). *B. vulgatus* and *E. coli* are suspected to drive insulin resistance via branched-chain amino acids (Leite et al., 2017; Pedersen et al., 2016). Interestingly, MS increased *Bacteroides spp.* and especially the species *B. vulgatus* as observed in type 2 diabetic patients, and directly associated with a loss of insulin sensitivity (Leite et al., 2017; Pedersen et al., 2016).

The microbiota dysbiosis observed at PND350 is not associated with intestinal permeability changes. Increased intestinal permeability is positively correlated with HOMA index in obese patients and (Teixeira et al., 2012) in high-fat diet (HFD) mice model (Cani et al., 2008). Furthermore, intestinal hyperpermeability is pointed out as a factor leading to endotoxemia that might contribute to low-grade inflammation and triggering metabolic disorders (Cani et al., 2007). A normal intestinal permeability in our model could be due to the absence of obesity. Indeed, intestinal hyperpermeability has been described in complex metabolic disorder i.e. type 2 diabetes associated with obesity in mice and human model but never in type 2 diabetes in lean individuals. In mouse model of HFD-induced intestinal hyperpermeability associated with an increase of HOMA index, restoring intestinal permeability by fish oil treatment or resolvin D1 did not improve HOMA index (Lam et al., 2015), suggesting that correcting intestinal permeability is not sufficient to ameliorate metabolic status. Cells isolated from small intestine and spleen of MS mice at PND350 secrete higher TNF α concentration in response to LPS *in vitro* stimulation compared to control mice. Interestingly, childhood victimization is correlated with higher plasmatic CRP levels in young adult in human (Baldwin et al., 2017). Until today, there is no consensus among scientists if low-grade inflammation is cause or consequence of metabolic disorder. On one hand, obesity leads to low-grade inflammation through the exceeding production of inflammatory molecules by white adipose tissue (Gregor and Hotamisligil, 2011) whereas, on the other hand, endotoxemia can induce obesity and insulin resistance (Cani et al., 2007). MS also decreased IgG in feces adding evidence to impaired intestinal barrier

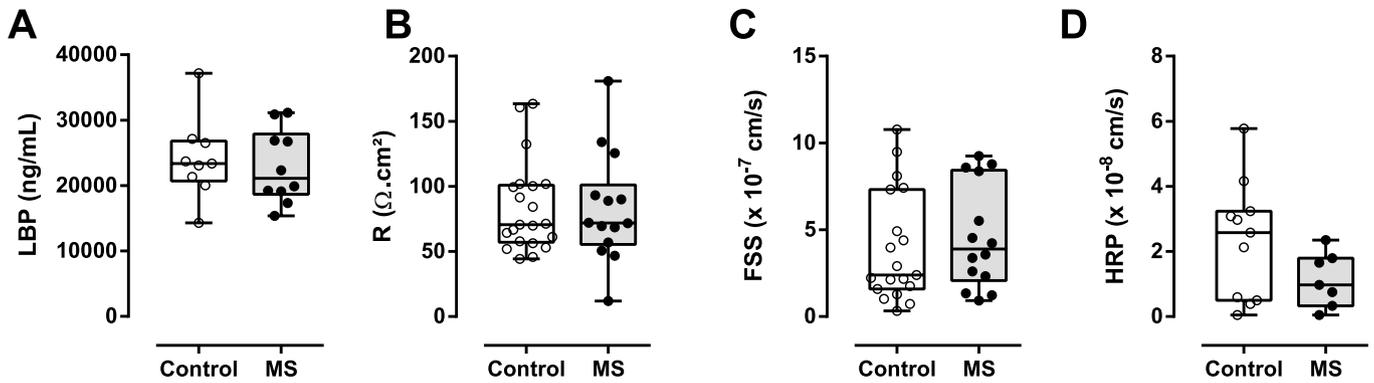


Fig. 4. MS had no consequences on intestinal permeability. (A) LBP concentration in plasma (ng/mL) n = 9–10. (B–D) *Ex vivo* Ussing chambers experiment in jejunum (B) Electrical resistance ($\Omega \times \text{cm}^2$), n = 14–21. (C) Paracellular permeability to FSS ($\times 10^{-7}$ cm/s), n = 14–19. (D) Transcellular permeability to HRP ($\times 10^{-8}$ cm/s), n = 7–11.

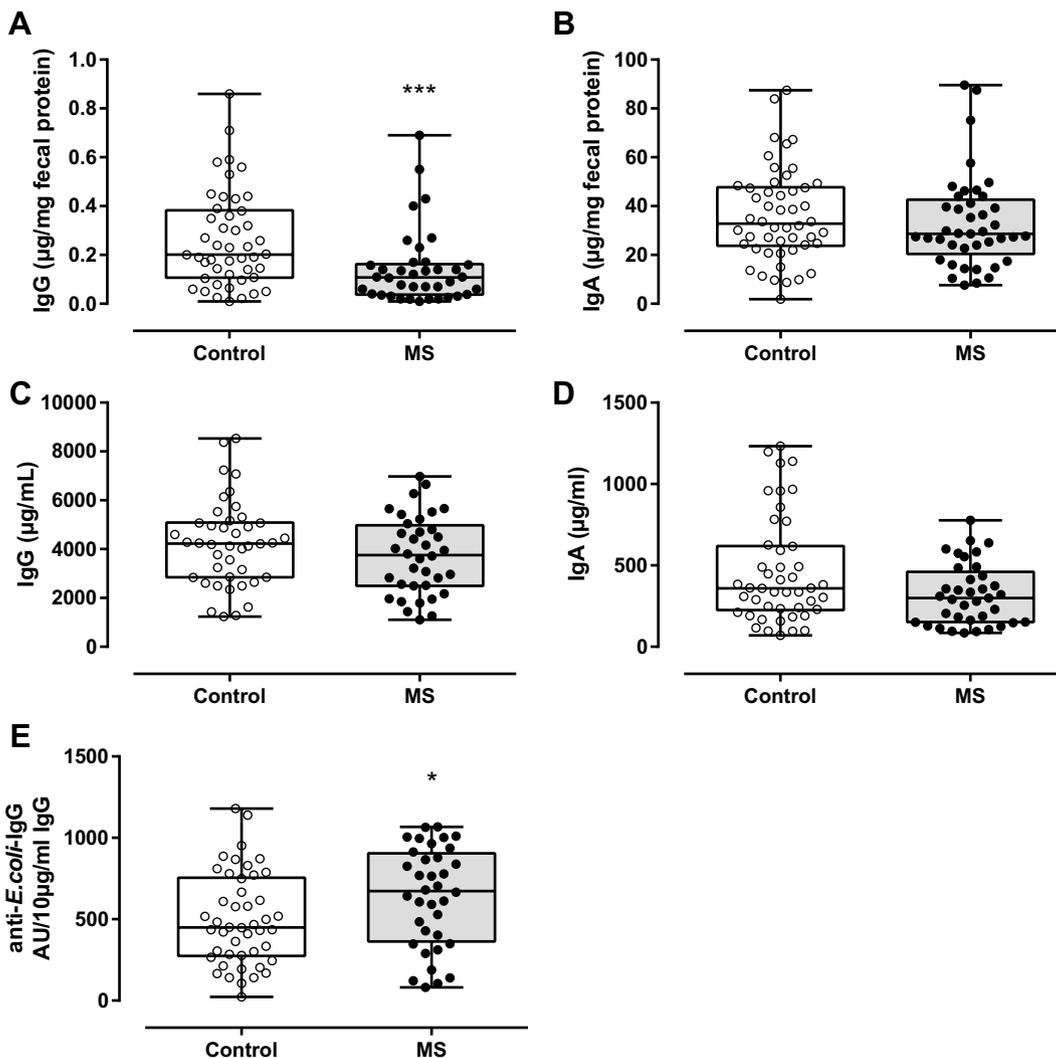


Fig. 5. MS decreased fecal IgG and increased anti-*E. coli* IgG in plasma. (A) Fecal IgG content ($\mu\text{g}/\text{mg}$ fecal protein), n = 38–46, Mann Whitney test, ***p < 0.001. (B) Fecal IgA content ($\mu\text{g}/\text{mg}$ fecal protein), n = 41–50. (C) Plasma IgG concentration ($\mu\text{g}/\text{mL}$), n = 36–42. (D) Plasma IgA concentration ($\mu\text{g}/\text{mL}$), n = 37–46. (E) Plasmatic IgG against *E. coli* lysate (arbitrary units/10 $\mu\text{g}/\text{mL}$ IgG), n = 36–46, Mann Whitney test, *p < 0.05.

despite no modification of intestinal permeability. This defect is also reflected by higher TNF α in response to LPS in siLP and spleen, as well as by higher anti-*E. coli* IgG in plasma. Interestingly, IgG against commensal antigens were increased in diabetic patients (Mohammed et al., 2012).

In our model, intestinal IL-17 and IL-22 and systemic IL-17 secretions were impaired after TCR stimulation. The defect of IL-17 and IL-22 secretion is not due to modification of populations, but rather to a malfunction of Th17 and Th22 cells. The observed decrease of IL-17 and IL-22 secretion is of particular interest since it was also observed in

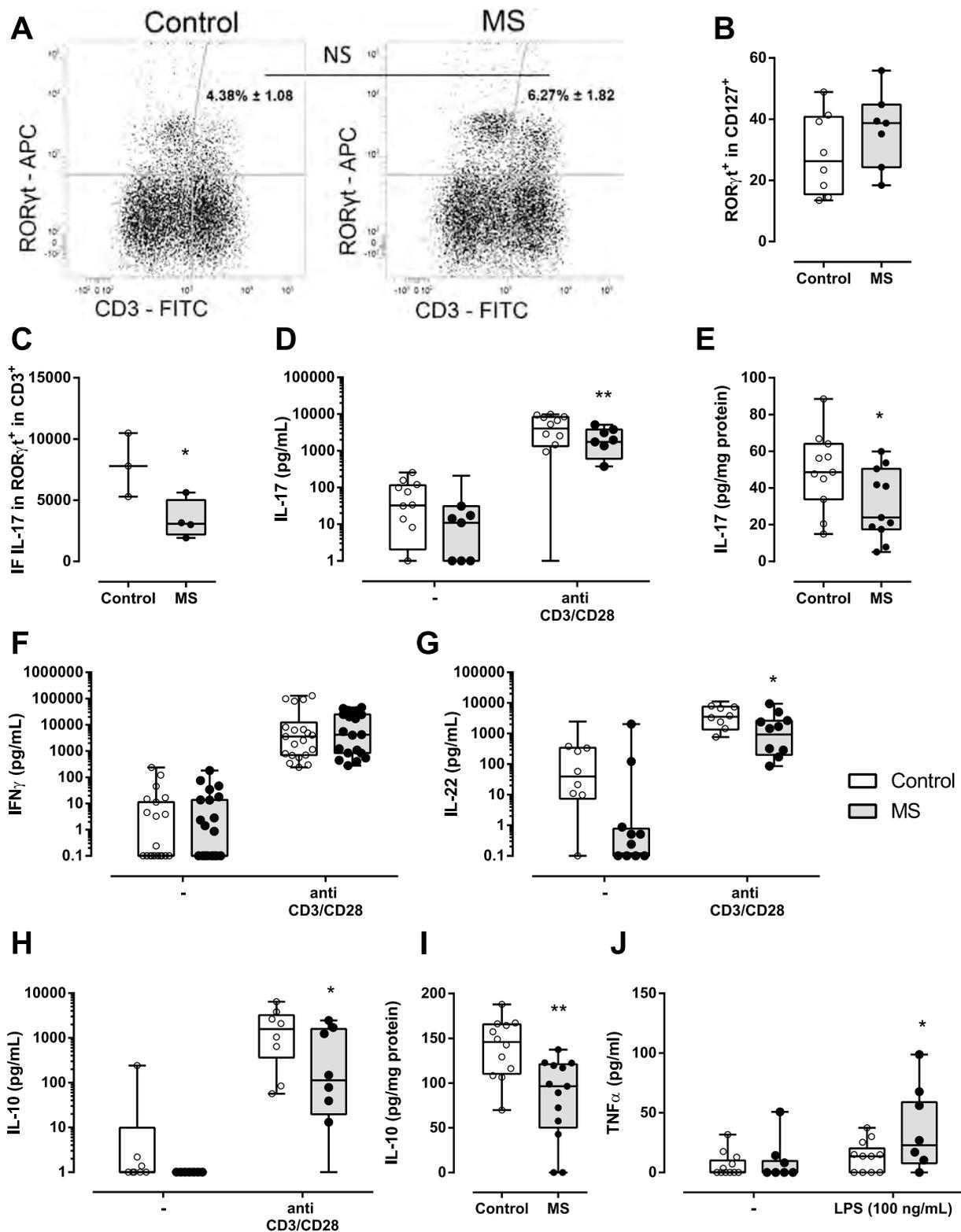


Fig. 6. MS impaired IL-17 and IL-22 secretion and increased TNF α in small intestine *lamina propria* (siLP). (A) Representatives dot plots of CD3⁺ROR γ t⁺ cells of siLP, n = 3–4, unpaired t-test. (B) ILC3 in isolated siLP (ROR γ t⁺ in CD127⁺), n = 8–7. (C) Median fluorescence intensity of IL-17 on T helper 17 cell population (CD3⁺ROR γ t⁺ cells) of siLP, n = 3–4, unpaired t-test, *p < 0.05. (D, F–H) Cytokine secretion in siLP cell culture after 72 h without or with anti-CD3/CD28 (5 μ g/mL) stimulation. (D) IL-17, n = 11–12. (F) IFN γ , n = 23. (H) IL-10, n = 9–10. (G) IL-22, n = 10–12, RM two-way ANOVA, *p < 0.05, **p < 0.01. (E, I) Cytokine concentration (pg/mg protein) in jejunal tissue. (E) IL-17, n = 11 (I) IL-10, n = 12–13, unpaired t-test, *p < 0.05, **p < 0.01. (J) TNF α secretion in siLP cell culture after 16 h with or without LPS (100 ng/mL) stimulation, n = 10–13, RM two-way ANOVA, * p < 0.05.

other models of hyperglycemia induced by nutritional challenge and genetically engineer. Indeed, HFD leading to obesity and type 2 diabetes are associated with a decrease of intestinal Th17 and Th22

populations (Garidou et al., 2015; Hong et al., 2017; Wang et al., 2014). Furthermore, ROR γ t KO mice deficient for Th17/22 cells present a mild glucose intolerance (Garidou et al., 2015). The direct or indirect

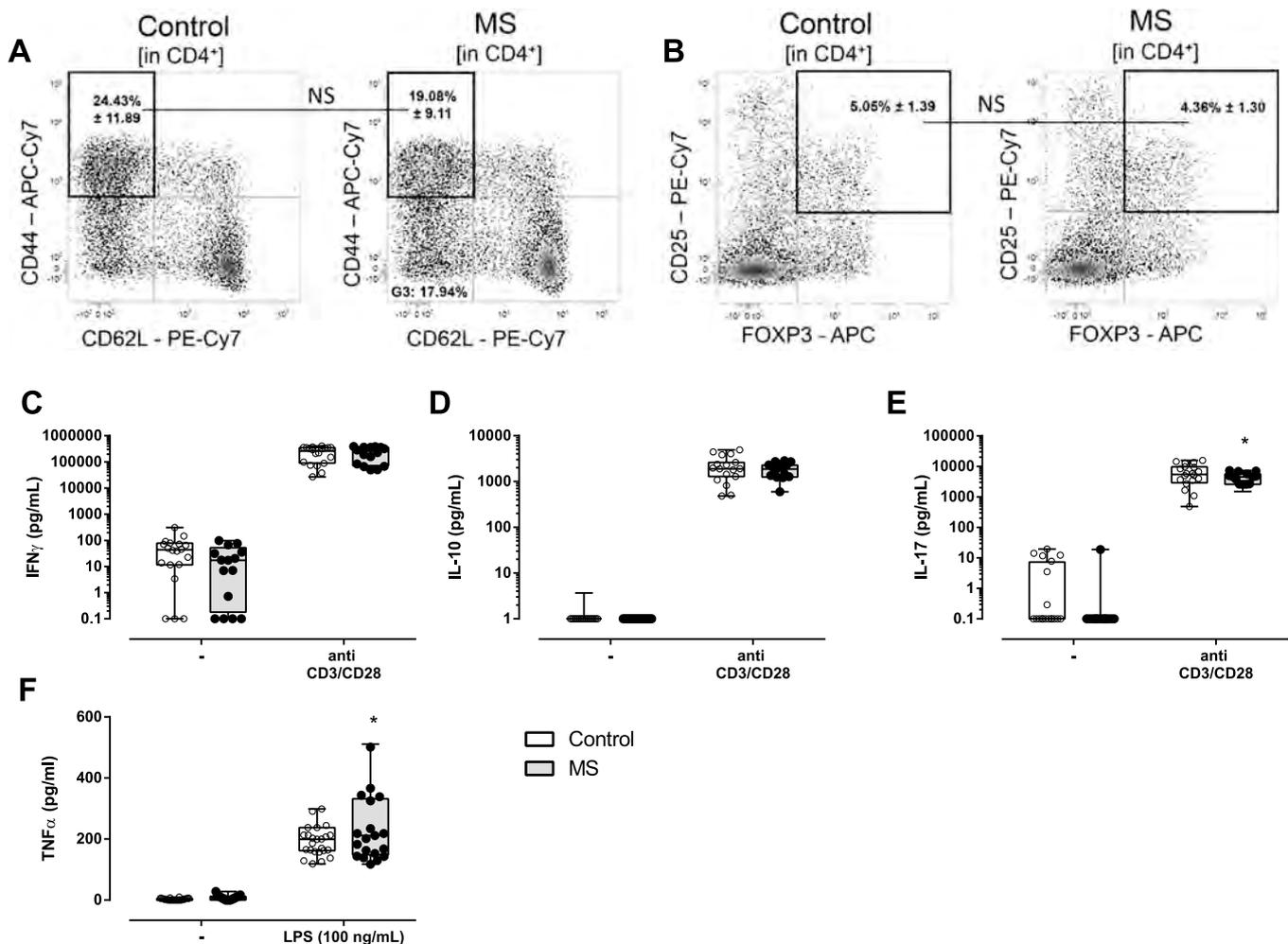


Fig. 7. MS impaired IL-17 secretion and increased TNF α in spleen. (A) Representative dot plots of activated T cell population in spleen (CD44^{high} CD62L^{low} in CD4⁺), n = 5–7. (B) Representative dot plots of regulatory T cell population in spleen (CD25⁺ Foxp3⁺ in CD4⁺), n = 5–7. (C)–(E) Cytokine secretion in primary cell culture of splenocytes after 72 h without or with anti-CD3/CD28 (5 μ g/mL) stimulation, RM two-way ANOVA, **p < 0.01. (C) IFN γ , n = 16–20. (D) IL-10, n = 16–19. (E) IL-17, n = 15–20. (F) TNF α secretion in primary cell culture of splenocytes after 16 h with or without LPS (100 ng/mL) stimulation, RM two-way ANOVA, *p < 0.05.

increase of IL-22 moderates metabolic disorder induced by HFD (Wang et al., 2014; Zou et al., 2017) and restores microbiota (Gulhane et al., 2016; Zou et al., 2017) and intestinal barrier (Gulhane et al., 2016).

Taking together the results of this study show that MS in wild type mice under normal diet induces glucose intolerance associated with a loss of insulin sensitivity. Interestingly, glucose intolerance in MS model is associated with a decrease of intestinal IL-17 and IL-22 secretion as previously observed in studies of HFD-induced metabolic disorders. Consequently, MS induce the same adverse effects as HFD, without obesity and intestinal hyperpermeability. Finally, this study highlights early life stress as a risk factor for metabolic disorders development independently of nutritional challenge and early life period as critical time window for appropriate establishment of immune system and metabolism.

Acknowledgements

We thank the platform GenoToul Anexplor, Toulouse, France for plasma analysis, M2C, Toxalim, Toulouse, France for assistance in cytometry analysis. We are grateful to the Get-platform teams (Genomic & Transcriptomic, TRIX & PlaGe, Toulouse, France) for 16S rDNA libraries and sequencing, the Genotoul bioinformatics platform Toulouse Midi-Pyrénées and Sigena group for providing help and storage resources thanks to Galaxy instance <http://sigena-workbench.toulouse.inra.fr>.

Maithé Taubert, Childrens Hospital Purpan, Toulouse, France for scientific advice.

Funding

Hanna Ilchmann-Diounou is a grant recipient of French Ministry of Research and European Society of Pediatric Endocrinology travel grant.

Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbi.2019.04.025>.

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SUPPLEMENTARY DATA

Early life stress induces type 2 diabetes-like features in ageing mice

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SUPPLEMENTARY METHODS

16S rRNA Amplification and Amplicon Sequencing

The microbial 16S rRNA gene was amplified during the first PCR step with adapter fusion primers targeting the V3 to V4 regions (corresponding to a 460-bp region of *Escherichia coli* 16S rRNA gene, GenBank number J01695). The forward PCR primer 5'**CTT TCC CTA CAC GAC GCT CTT CCG ATC TAC GGR AGG CAG CAG**3' was a 43-nucleotide fusion primer consisting of the 28-nt illumina adapter (designed by bold font) and the 14-nt broad range bacterial primer 343F (Liu et al., 2007). The reverse PCR primer 5'**GGA GTT CAG ACG TGT GCT CTT CCG ATC TTA CCA GGG TAT CTA ATC CT**3' was a 47-nucleotide fusion primer consisting of the 28-nt illumina adapter (designed by bold font) and the 19-nt broad range bacterial primer 784R (Andersson et al., 2008).

The PCR mix contained MTP Taq DNA polymerase (SIGMA, 0,05 U/μl), 200 μM of each DNTP (SIGMA, premix), and 0,5 μM of each primer. After initial denaturation at 94°C for 60 sec in CFX-96 Thermal Cycler (Bio-Rad), 30 cycles were run with 60 sec denaturation at 94°C, 60 sec annealing at 65°C and 60 sec at 72°C. Round ended with 10 min extension at 72°C. Pooled amplicon libraries were sequenced employing an Illumina MiSeq (2 x 250 bp) at the GeT-PlaGe platform in Toulouse (France). Amplification quality (length, quantity and specificity) was verified using the Agilent 2200 TapeStation system (High sensitivity D1000 ScreenTape Assay) and AATI Fragment Analyser at the GeT (Genomic and Transcriptomic, TRIX and PlaGe) platforms in Toulouse. The quality of the run was checked internally using PhiX, and then each pair-end sequences were assigned to its sample with the help of the previously integrated index.

Microbiome 16S Data Analysis

High quality filtered reads were further processed using FROGS pipeline (Find Rapidly OTU with Galaxy Solution (Escudié et al., 2017)) to obtain OTUs and their respective taxonomic assignment thanks to Galaxy instance (<http://sigenae-worbench.toulouse.inra.fr>): Initial FROGS pre-process step allowed to select overlapped reads with expected length without N. Swarm clustering method (Mahé et al., 2014) was applied by using a first run for denoising with a distance of 1 and then a second run for clustering with an aggregation maximal distance of 3 on the seeds of first Swarm. Putative chimera were removed using Vsearch combined to cross-validation (GitHub repository. Doi 10.5281/zenodo.15524).

Cluster abundances were filtered at 0,005% (Bokulich et al., 2013) and/or had to be present at least in 2 samples, yielding to 316 clusters corresponding to 308625 final valid reads. Between 13 339 and 22 656 valid sequences per sample were counted (no significant difference between groups was noticed; $p=0.421$, unpaired t test with equal SD). 100% of clusters were affiliated to OTU by using a silva128 16S reference database and a taxonomic multi-affiliation procedure (Blast+ (Camacho et al., 2009) with equal multi-hits). Since rarefaction has shown to result in high rates of false positive tests for differential abundance (McMurdie and Holmes, 2014), counts were not rarefied.

OTU prevalence, rarefaction curves were plotted for each sample by using Phyloseq package (v 1.19.1, Figure 3, Supplementary Figure 1A). Within sample community, alpha diversity was assessed by Chao-1 index. Divergence in community composition between samples was qualitatively and quantitatively assessed by calculating both unweighted (an investigation into the presence and absence of taxa) and weighted (which takes relative abundances of taxa in account) Unifrac distance matrices. Unconstrained ordination was visualized using multidimensional scaling (MDS) and hierarchical clustering (complete linkage combined with Unifrac distance, Supplementary Figure 1A) and compared using Adonis test (9999 permutations).

In order to determine features at each phylogenetic rank that characterize fecal microbiota of PND350 mice in response to maternal separation, LefSe algorithm (Segata et al., 2011) was performed with an alpha value of 0.05 (Kruskall-Wallis non parametric pairwise comparisons) and a threshold on the logarithmic LDA score for discriminative features of 2.

OTUs were agglomerated at the species rank before univariate differential abundance of taxa was obtained using a negative binomial noise model for over dispersion as implemented in the R package DESeq2 (v 1.14.1) (Love et al., 2014; McMurdie and Holmes, 2014). Taxa were considered significantly differentially

abundant between treatments if their adjusted p-value was below 0.05.

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LEGENDS SUPPLEMENTARY FIGURES

Supplementary Figure 1: MS induces gut dysbiosis in offspring at PND350. (A) Prevalence per OTUs in samples associated with hierarchical clustering based on the Unifrac distances with Ward linkage. (B) Histogram of the LDA scores computed for affiliated OTUs that were found differentially abundant between MS and control mice. The magnitude of the LEfSe scores higher than 3 and $p < 0.05$ are displayed. Differences are represented in the color of the most abundant class. (C) Relative abundance of taxa agglomerated at the species rank that were found differentially abundant using Deseq2 analysis, adjusted p-value < 0.05 .

Additional data

Lysozyme activity is resolved at PND350

At PND50 MS induced in male mice intestinal barrier dysfunction, namely intestinal hyperpermeability, decreased fecal lysozyme activity and increased fecal IgA (Riba et al., 2018). In my article (Ilchmann-Diounou et al., 2019) we showed, that intestinal permeability is restored at PND350. We wondered if lysozyme activity in PND350 mice is still modified. Feces were collected and frozen at -80°C . Activity of lysozyme against peptidoglycan was determined using the EnzChek® Lysozyme Assay Kit (Molecular probes, life technology).

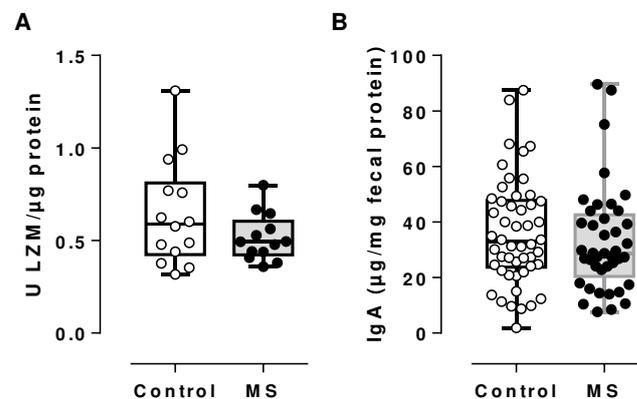


Figure 23 Resolved parameters of intestinal barrier dysfunctions in maternal separated (MS) or control C3H/HeN mice at post-natal day (PND) 350 (A) Lysozyme (LZM) activity in fecal supernatant (U/μg fecal protein) of feces measured by ELISA. (B) fecal IgA concentration (μg/mg fecal protein) measured by ELISA.

At PND350 fecal lysozyme activity in MS and control mice were similar. As a consequence, decreased lysozyme activity at PND50 is resolved in time (**Figure 23A**). Fecal IgA were similar between MS and control at PND350 (**Figure 23B**) but increased at PND50. These are arguments that intestinal barrier dysfunction resolves with time. One can hypothesize that, the effects of MS on immune-metabolism are due to a delay in intestinal barrier development. However, even though intestinal permeability and lysozyme activity are corrected, the intestinal immune system is not. Indeed, fecal IgG were diminished by MS at PND350 (Ilchmann-Diounou et al., 2019). Furthermore, intestinal low-grade inflammation is persisting TNF α secretion in response to LPS stimulation is increased and diminished IL-17 and IL-22 in siLP show that intestinal immune system in MS mice is distinct from control. The delayed maturation of intestinal barrier namely delayed gut closure and delayed lysozyme activity could be responsible for a wrong priming of the immune system, leading to long-lasting intestinal and systemic low-grade inflammation.

However, interventional studies are needed to prove this hypothesis.

Microbiota dysbiosis is different between PND50 and PND350

We were interested in evolution of microbiota in our model over the time of the experiment. We wondered, if microbiota dysbiosis at PND50 is similar to microbiota dysbiosis at PND350.

We confirmed that the bacterial signature associated with MS was specific of PND350 mice since bacterial signature observed in PND50 mice was distinct from that of PND350 mice (**Figure 24A**) and not driven by pathobiont expansion (**Figure 24B**), unlike what we observed at PND350 (Ilchmann-Diounou et al., 2019).

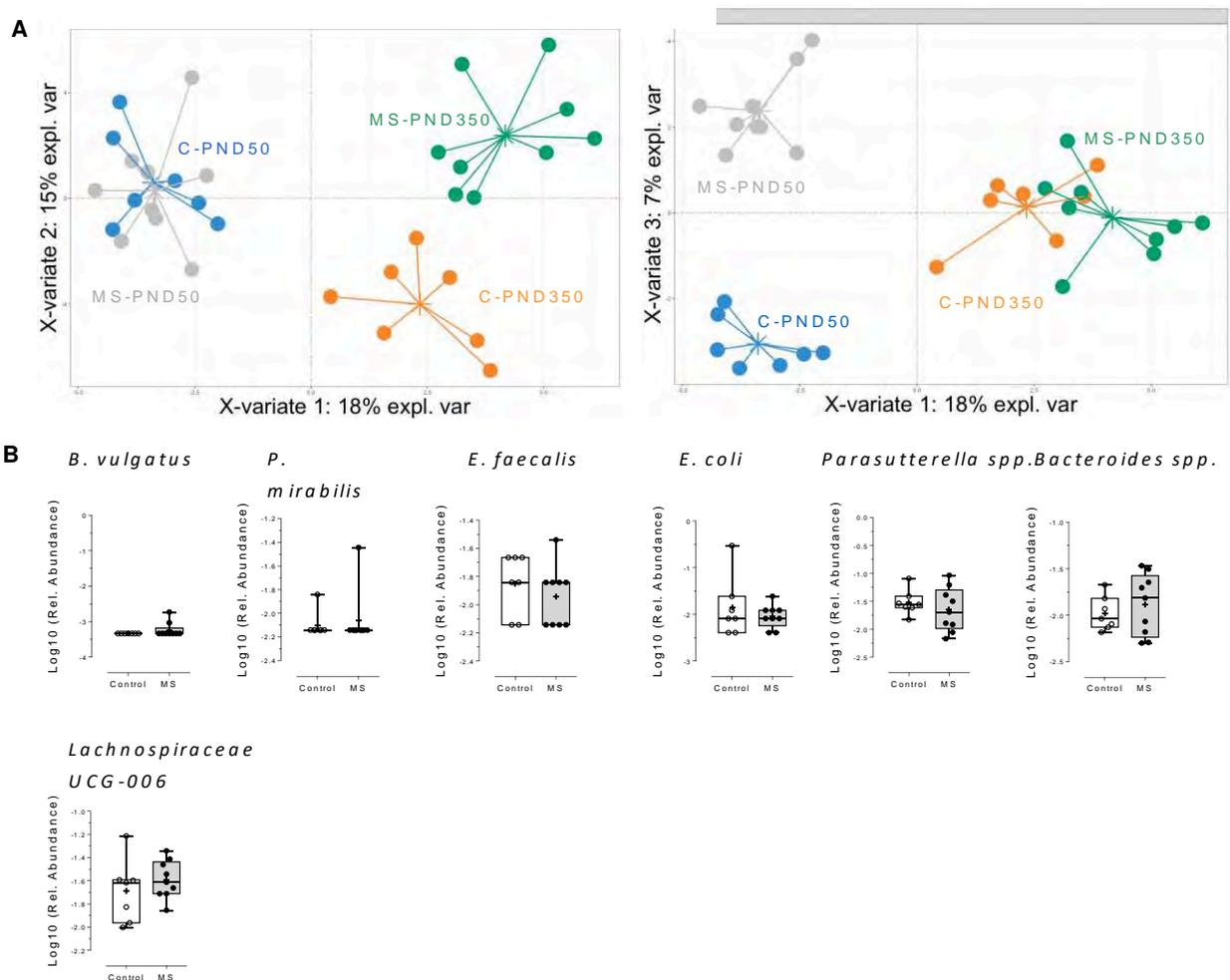


Figure 24 Bacterial taxonomic patterns that characterize fecal microbiota of PND350 mice and PND50 mice differ significantly. (A) PLS-DA score plots on the first three components (top panel: X-variates 1 and 2, bottom panel: X-variates 1 and 3) built from affiliated OTUs agglomerated at the species rank (74 taxa). Samples are classified into four classes: Control mice at PND50 (C-PND50), PND350 (C-PND350) and MS mice at PND50 (MS-PND50) or PND350 (MS-PND350). (B) Relative abundance of taxa agglomerated at the species rank in PND50 mice. Taxa displayed are not altered by MS at PND50 (adj-p >0.1) despite they were previously found differentially abundant using Deseq2 analysis in PND350 mice. (PLS-DA = Partial least square discriminant analysis; OUT = operational taxonomic unit)

**SECOND RESULT:
ROLE OF MICROBIOTA IN MS-INDUCED GLUCOSE INTOLERANCE
IN AGING MICE**

Work in progress

*In collaboration with Olier, Maïwenn; Ilchmann-Diounou, Hanna; Lencina, Corinne;
Guzylack-Piriou, Laurence; Balvay, Aurélie; Maudet, Claire; Foussier, Anne; Rabot, Sylvie;
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Microbiota dysbiosis is described to play a crucial role in the development of various non-communicable diseases, including metabolic disorders. The definition of dysbiosis proposed notably by Peterson and Round (Petersen and Round, 2014), is a disturbance in the microbiome structure that may consist in a loss of beneficial microorganisms, and/or expansion of pathobionts or harmful microorganisms.

Since microbiota is modified in our model of neonatal maternal separation (MS), and a tremendous amount of articles showed that microbiota dysbiosis is sufficient to induce metabolic disorder in germ-free recipient mice (Bäckhed et al., 2004; Turnbaugh et al., 2006), we wondered, if the observed effects of MS are triggered by gut microbiota dysbiosis. Germ-free mice are the recent gold standard to prove a causal relationship.

We set up collaboration with the germ-free platform ANAXEM at Micalis and Sylvie Rabot of the team Food, Gut Microbiota, Brain and Metabolic Diseases (AMIPEM) at Micalis, INRA Jouy en Josas, to carry out fecal microbiota transfer (FMT) of PND350 MS and control fecal samples into germ-free (GF) mice.

WORK IN PROGRESS

**ROLE OF MICROBIOTA IN MS-INDUCED GLUCOSE INTOLERANCE
IN AGING MICE**

COLLABORATIONS

Neuro-Gastroenterology and Nutrition team, Toxalim (Research Centre in Food Toxicology), Université de Toulouse, INRA, ENVT, INP-Purpan, UPS, Toulouse, France: Hanna Ilchmann, Maïwenn Olier, Sandrine Ménard

AMIPEM (Food, Gut Microbiota, Brain and Metabolic Diseases) INRA Jouy en Josas, France: Sylvie Rabet.

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INTRODUCTION

Intestinal microbiota is an important factor to intestinal homeostasis but more largely contribute to the host's wellbeing. Host and microbiota interact in a symbiotic relationship that benefits to both: host and microbiota (Bäckhed et al., 2005). Microbiota dysbiosis is described to play a crucial role in the development of various non-communicable diseases, including metabolic disorders. The definition of dysbiosis proposed notably by Peterson and Round (Peterson and Round, 2014), is a disturbance in the microbiome structure that may consist in a loss of beneficial microorganisms, and/or expansion of pathobionts or harmful microorganisms. The main issue remaining in microbiota study was to define the 'healthy' microbiota. This was the aim of the first phase of the National Institutes of Health Human Microbiome Project (NIH HMP), which started in 2007 (Turnbaugh et al., 2007). Then, the second phase (integrative HMP, iHMP or HMP2) aimed to focus on the role of microbiota on the onset of diseases including type 2 diabetes (T2D) (Zhou et al., 2019). Before iHMP, obesity and diabetes, were described to be associated with gut dysbiosis (Ley et al., 2005; Qin et al., 2012). The strength of iHMP in T2D is the following of participants (healthy and pre-diabetics) for 4 years (Zhou et al., 2019). This allows to associate microbiome profile to insulin sensitivity status that confirm microbiota dysbiosis associated to T2D and might contribute to early detection of T2D (Zhou et al., 2019). Those epidemiological studies need to be completed by mechanistic experiments to validate causative associations.

Neonatal maternal separation (MS) has been described to play a role in the development of metabolic diseases (Aya-Ramos et al., 2017; Ghosh et al., 2016; Ilchmann-Diounou et al., 2019). Indeed, MS induces type 2 diabetes-like features in aging, namely fasted hyperglycemia, glucose intolerance and insulin resistance, associated with intestinal and systemic low-grade inflammation, decreased intestinal IL-17 secretion and increased humoral response against microbiota. This immune-metabolic phenotype was associated with fecal dysbiosis (Ilchmann-Diounou et al., 2019).

We aimed to address the role of microbiota in MS-induced glucose intolerance. Germ free mice raised under axenic condition represent an interesting tool to answer this question. So, we set up a fecal microbiota transfer from old mice which underwent or not MS in early life into adult germ-free mice.

MATERIAL & METHODS

Mouse model

The donor mice, conventional, Specific and Opportunistic Pathogen Free (SOPF), C3H/HeN male mice, aged post-natal day (PND350), were raised in INRA Toulouse mouse house facilities. They are issue of an anterior experiment, experimental protocol and maternal separation (MS) procedure were previously described (Ilchmann-Diounou et al., 2019). Experimental protocol were approved by local Animal Care Use Committee (Comité d'Ethique de Pharmacologie-Toxicologie de Toulouse - Midi-Pyrénées, France) registered as N°86 at the Ministry of Research and Higher Education (N° 0029/SMVT), and conducted in accordance with the European directive 2010/63/UE. MS and control mice were kept at a constant temperature ($22 \pm 1^\circ\text{C}$) and maintained on a 12:12 h light/dark cycle (lights on 7h30 am). Normal diet (Harlan2018, Envigo, Gannat, France) and water were available *ad libitum*.

The germ-free C3H/HeN male were obtained locally from the germ-free rodent breeding facility of Anaxem (Germfree animal facilities of the Micalis Institute, France). They were housed in sterile Plexiglas isolators (Eurobioconcept, Paris, France). The germ-free status was monitored weekly by microscopic examination and aerobic and anaerobic cultures of freshly voided feces. All mice were kept in Macrolon cages (38 cm long, 22 cm wide, 21 cm high) containing sterile beddings made of wood shavings. They were given a free access to autoclaved tap water and a γ -irradiated (45 kGy) normal diet (Harlan2018, Envigo, Gannat, France). The isolators were maintained at 20–24°C and on a 12-h light/dark cycle (lights on at 07:30 a.m.). Experimental procedures were conformed to the European guidelines for the care and use of laboratory animals. They were carried out in accordance with the recommendations of and approved by (approvals #10724) the ethics committee of the INRA Research Center at Jouy-en-Josas (ethics committee named Comethea, registered by the French Ministry in charge of Research since 2011/06/30 with reference number 45).

Colonization of Germ Free mice

Fecal homogenate of PND350 maternal separated (MS) and control mice were prepared as follows. A pool of feces collected from 14 mice was suspended at 1/100 in Ringer solution supplemented with 0.05% (w/v) L-cysteine (HCl) and 10% skimmed milk and frozen at -80°C prior to use.

PND150 GF mice were orally inoculated with a 1:100 dilution of fecal homogenate from PND350 MS mice, PND350 control mice or vehicle (Ringer solution supplemented with 0.05%

(w/v) L-cysteine (HCl) and 10% skimmed milk). Sixteen weeks after colonization, mice were used for analysis. Mice that received fecal homogenate from PND350 MS mice are named GFMS; from PND350 control mice are named GFC whereas mice receiving the vehicle are named GFM (Milk added to Ringer solution) mice. All mice had same age at analysis (150 days + 16 weeks = 262 days).

Fecal microbiota composition analysis

Total microbial genomic DNA was obtained from stool samples using the ZR Fecal DNA Miniprep™ (Zymo Research, Ozyme SAS, Montigny-le-Bretonneux, France) and DNA quantity was determined using a TECAN Fluorometer (Qubit® dsDNA HS Assay Kit, Molecular Probes, Thermofisher Scientific, Montigny le Bretonneux).

16S rRNA Amplification and Amplicon Sequencing

The microbial 16S rRNA gene was amplified during the first PCR step with adapter fusion primers targeting the V3 to V4 regions (corresponding to a 460-bp region of *Escherichia coli* 16S rRNA gene, GenBank number J01695). The forward PCR primer 5'**CTT TCC CTA CAC GAC GCT CTT CCG ATC TAC** GGR AGG CAG CAG3' was a 43-nucleotide fusion primer consisting of the 28-nt illumina adapter (designed by bold font) and the 14-nt broad range bacterial primer 343F (Liu et al., 2007). The reverse PCR primer 5'**GGA GTT CAG ACG TGT GCT CTT CCG ATC TTA** CCA GGG TAT CTA ATC CT3' was a 47-nucleotide fusion primer consisting of the 28-nt illumina adapter (designed by bold font) and the 19-nt broad range bacterial primer 784R (Andersson et al., 2008).

The PCR mix contained MTP Taq DNA polymerase (SIGMA, 0,05 U/μl), 200 μM of each dNTP (SIGMA, premix), and 0,5 μM of each primer. After initial denaturation at 94°C for 60 sec in CFX-96 Thermal Cycler (Bio-Rad), 30 cycles were run with 60 sec denaturation at 94°C, 60 sec annealing at 65°C and 60 sec at 72°C. Round ended with 10 min extension at 72°C. Pooled amplicon libraries were sequenced employing an Illumina MiSeq (2 x 250 bp) at the GeT-PlaGe platform in Toulouse (France). Amplification quality (length, quantity and specificity) was verified using the Agilent 2200 TapeStation system (High sensitivity D1000 ScreenTape Assay) and AATI Fragment Analyser at the GeT (Genomic and Transcriptomic, TRIX and PlaGe) platforms in Toulouse. The quality of the run was checked internally using PhiX, and then each pair-end sequences were assigned to its sample with the help of the previously integrated index.

Microbiome 16S Data Analysis

High quality filtered reads were further processed using FROGS pipeline (Find Rapidly OTU with Galaxy Solution (Escudié et al., 2018)) to obtain OTUs and their respective taxonomic assignment thanks to Galaxy instance (<http://sigenae-worbench.toulouse.inra.fr>): Initial FROGS pre-process step allowed to select overlapped reads with expected length without N. Swarm clustering method (Mahé et al., 2014) was applied by using a first run for denoising with a distance of 1 and then a second run for clustering with an aggregation maximal distance of 3 on the seeds of first Swarm. Putative chimera were removed using Vsearch combined to cross-validation (GitHub repository. Doi 10.5281/zenodo.15524). Cluster abundances were filtered at 0,005% (Bokulich et al., 2013) and/or had to be present at least in 2 samples. 100% of clusters were affiliated to OTU by using a silva128 16S reference database and a taxonomic multi-affiliation procedure (Blast+ (Camacho et al., 2009) with equal multi-hits). Diversity indexes of bacterial community, as well as ordinations were computed using the Phyloseq package (v 1.19.1) in RStudio software (McMurdie and Holmes, 2012; McMurdie and Holmes, 2013; R Development Core Team, 2011). Divergence in community composition between samples was assessed by calculating unweighted (an investigation into the presence and absence of taxa) Unifrac distance matrice. Unconstrained ordination was visualized using multidimensional scaling (MDS) and compared using Adonis test (9999 permutations).

Oral glucose tolerance test (OGTT)

OGTT were performed in mice after 16 h-fasted during night. For OGTT, mice were gavaged with 2 mg glucose per g of bodyweight. Blood glucose levels were monitored from the tip of the tail vein with a glucose meter (Johnson & Johnson, Issy-les-Moulineaux, France) at -30, 0 (glucose gavage), 15, 30, 60, 90 and 120 min.

For fasted blood glucose, mice were fasted 16-h overnight.

Intestinal permeability in Ussing chambers

Intestinal permeability was assessed in jejunal fragments on Ussing chambers. Briefly, jejunal fragments were mounted in Ussing chambers (Physiologic Instruments, San Diego, CA, USA). Tissues were bathed 2h with oxygenated thermostated Krebs's solution (Sigma, Saint Quentin Fallavier, France). Fluorescein Sodium Salt 40 µg/ml (FSS 376Da; Sigma) was added to mucosal compartment as para-cellular marker of intestinal permeability.

Fluorescence intensity was measured 485nm/525nm using an automatic Infinite M200 microplate reader (Tecan, Männedorf, Switzerland). Epithelial permeability to FSS was

determined by measuring the fluorescence intensity (FI) 485 nm/525 nm using an automatic Infinite M200 microplate reader. Permeability was calculated as the ratio of flux/concentration, and expressed as cm/second.

Immune cells isolation

Splenocytes were isolated through a 70- μ m nylon mesh and suspended in PBS 1%-KO SR serum (Gibco, Thermofisher Scientific).

Isolated cells from Small Intestines (si) *lamina propria* (siLP) were obtained as follow: si were washed in cold PBS, incubated in PBS 3 mM EDTA (Sigma), washed in warm PBS, digested with 100 U/mL of collagenase (Sigma) in DMEM 20% FCS and finally purified on Percoll (Sigma).

Primary cell culture

Isolated cells from spleen and siLP were seeded at 2×10^6 cells per well in the presence or absence of 5 μ g/mL hamster anti-mouse CD3 (BD) and hamster anti-mouse CD28 (BD) coated wells. Supernatants were collected after 72h.

Cytokines measurement

Cytokines were measured in supernatant of primary cell culture, or jejunal fragments suspended in RIPA buffer (0.5% deoxycholate, 0.1% SDS and 1% Igepal in TBS) containing complete anti protease cocktail (Sigma). Jejunal protein concentrations were measured using BCA uptima kit (Interchim, Montlucon, France). IL-17, IFN γ , and IL-22 in supernatant or lysate of jejunal fragments were assayed using commercial ELISA kits (R&D Systems, Lille, France).

Humoral response in feces and plasma

Plates were coated with 5 μ g/ml of sheep anti-mouse IgA (Sigma) or goat anti-mouse IgG (SouthernBiotech, Cliniscience, Nanterre, France), incubated with plasma, detected with 1.5 μ g/ml HRP-conjugated goat anti-mouse IgA (Sigma) or goat anti-mouse IgG (SouthernBiotech). HRP was revealed using TMB and the reaction was stopped with H₂SO₄ before reading at 450 nm using automatic Infinite M200 microplate reader.

Immunoglobulin specificity against commensal E. coli

Maxisorp 96-wells plates were coated with 5 μ g/ml of protein from C3H/HeN isolated *E. coli* lysate (being used as representative bacteria of the intestinal microbiota), incubated with plasma (10 μ g/mL IgG or 30 μ g/mL IgA), and revealed as above-mentioned. Results were

expressed as arbitrary units (AU) per 10 µg/mL of IgG or AU per µg/mL IgA, in comparison with a standardized immune serum. The *E. coli* lysate was prepared as previously described (Riba et al., 2018).

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.04 (GraphPad Software, La Jolla, CA, USA). Results for single comparisons were displayed as box plots [min to max] and analyzed using either one-way ANOVA, following Dunnett's multiple comparison test (parametric) or Kruskal-Wallis test, following Dunn's multiple comparison test (non-parametric) in case of different standard deviations (Brown-Forsythe test). Multiple conditions in OGTT and cytokine measurements were displayed either as kinetics with SEM or box plots [min to max] and compared per family by Tukey multiple comparison posttest after a significant repeated measures (RM) two-way ANOVA. Differences were considered significant for $P < 0.05$.

RESULTS

Colonization led to distinct microbiota in GFC and GFMS mice

We confirmed by 16S rRNA gene sequencing that inoculation of GF mice with microbiota from MS (IMS) or control (IMT) mice lead 16 weeks after colonization to recipient mice with distinct microbiota (GFC and GFMS) (**Figure 1**). Indeed, GFM group was distant from GFC and GFMS along axis 1 in multidimensional scaling (MDS) of UniFrac distances. GFC and GFMS groups could be separated along axis 2. Microbiota of recipient GFC is similar to inoculum coming from control donor mice and microbiota of recipient GFMS is similar to inoculum coming from MS donor mice.

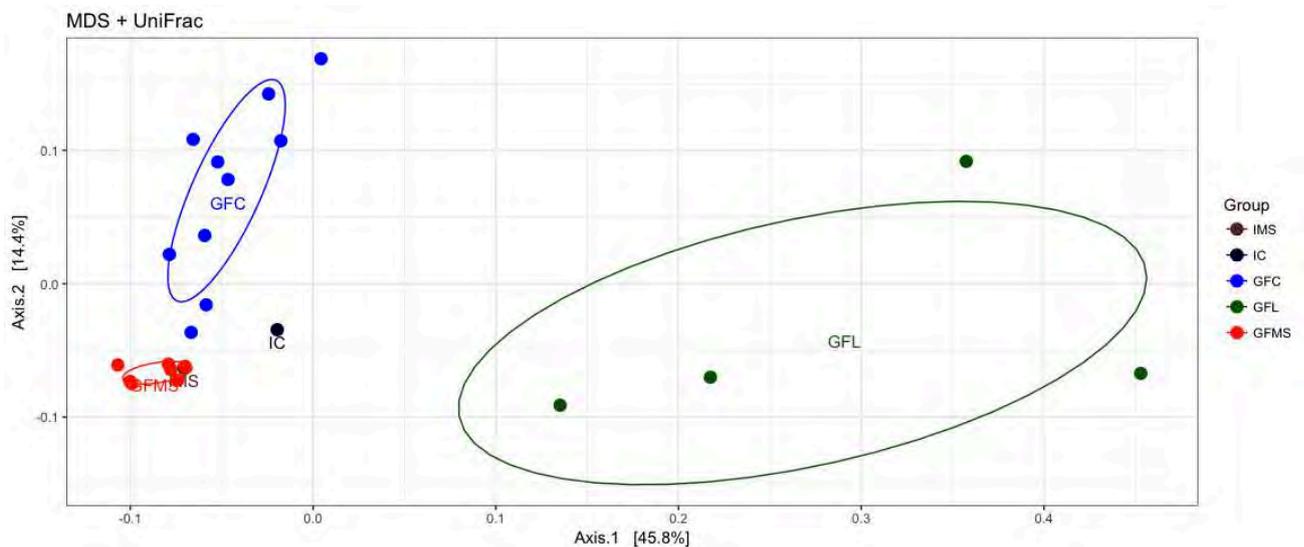


Figure 1 First two axes of a multidimensional scaling (MDS) of UniFrac distances from 16S rRNA gene sequencing of GFM (green), GFC (blue) and GFMS (red); inoculum of MS mice (IMS-brown), inoculum of control mice (IC-black).

Fecal microbiota transfer of MS donor failed to induce glucose intolerance in recipient

In order to test our hypothesis involving a role of MS microbiota driving glucose intolerance in aging mice (Ilchmann-Diounou et al., 2019), we looked at glucose metabolism in mice receiving either microbiota from MS (GFMS) or control mice (GFC) or the vehicle (GFM). Fecal microbiota transfer (FMT) of MS microbiota in recipient mice (GFMS) did not, after 16 weeks, increase fasted blood glucose compared to FMT of control microbiota (GFC) (**Figure 2A**). Glucose intolerance, as measured during oral glucose tolerance test (OGTT), was not induced by FMT of MS microbiota (**Figure 2B-C**). However, FMT of microbiota from MS and control mice induced glucose intolerance compared to mice receiving the vehicle (**Figure 2B-C**). In summary, FMT of MS mice was not sufficient to transfer MS metabolic phenotype observed at PND350.

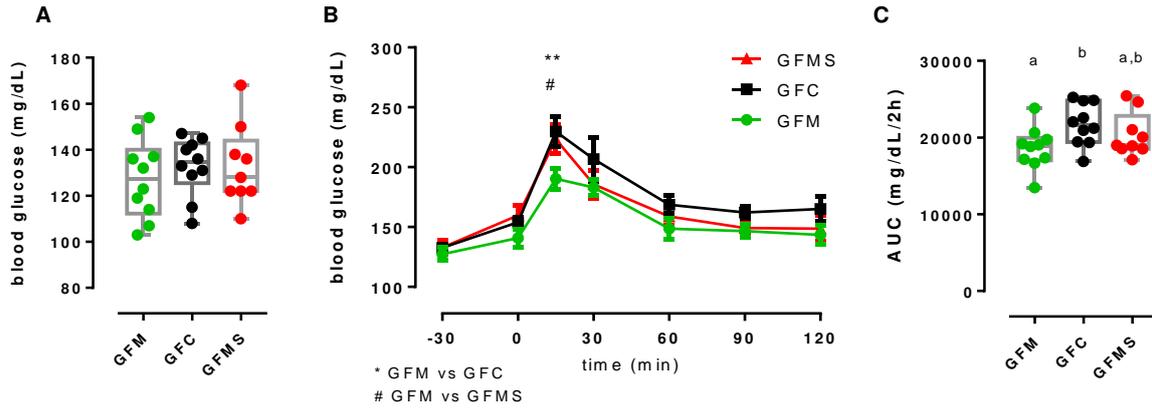


Figure 2 (A) 16h-fasted blood glycaemia. (B) Oral glucose tolerance test in 16h-fasted mice, blood glucose (mg/dL), two-way ANOVA, ** $p < 0.01$ GFC in comparison to GFM, # $p < 0.05$ GFMS in comparison to GFM. (C) Area under the curve (mg/dL/2h), one-way ANOVA, same letter = no statistical difference, different letters = significant differences.

Fecal microbiota transfer of MS donor failed to decrease IL-17 secretion in small intestine lamina propria

In our previous study, MS-induced glucose intolerance was associated with a decrease of IL-17 secretion in small intestine *lamina propria* (siLP). Even though FMT of MS microbiota in GFMS was not sufficient to induced glucose intolerance, we wonder if it could lead to the defect of IL-17 secretion in siLP. FMT of MS microbiota in GFMS did not decrease IL-17 concentration in jejunum nor IL-17 secretion in response to T cell receptor (TcR) stimulation in siLP cells (**Figure 3A-B**) compared to GFC or GFM.

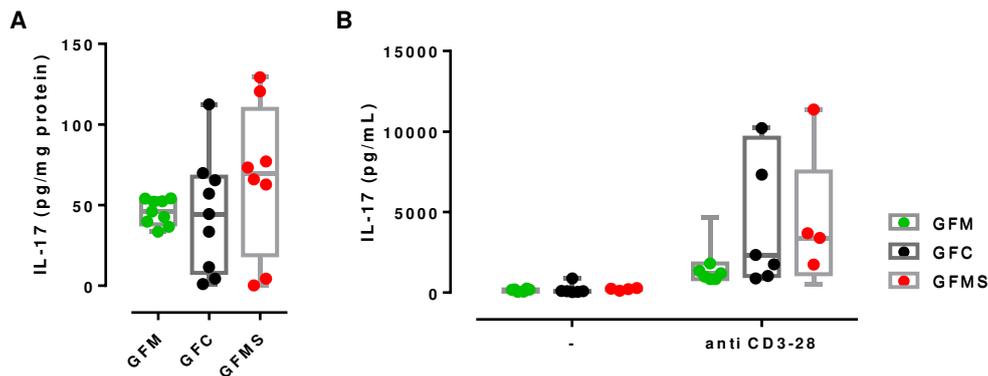


Figure 3 (A) IL-17 concentration (pg/mg protein) in lysate of small intestine (SI). (B) Primary cell culture of small intestine lamina propria (siLP). IL-17 concentration in cell culture supernatant (pg/mL) after 72h incubation without or with anti-CD3/CD28 stimulation.

In summary, our FMT protocol allowed to colonize GF recipient mice with MS (GFMS) and control microbiota (GFC) that are similar to inoculum from donor mice and as such led to different microbiota between GFMS and GFC. However, FMT of MS microbiota in GFMS ,

even after 16 weeks, was not sufficient to reproduce MS-induced phenotype, namely fasted hyperglycemia, glucose intolerance and defect of IL-17 secretion in small intestine.

We choose to make the most of this work by exploiting the results in a different way. Indeed, we took advantage of the obtained results to study the long-term effects of colonization (after 16 weeks) of GF adult mice with microbiota of aging (PND350) control mice on different parameters, notably intestinal permeability, intestinal and systemic immune response and glucose metabolism. Indeed, at our knowledge, colonization effects are principally measured in a short time window after colonization. We analyzed intestinal barrier, immune response and metabolism 16 weeks after colonization.

In the following part, results are presented and compared between GF, recipient (previous GFC) and donor mice (previous control).

What are the long-term effects of fecal microbiota transfer in adult germ-free mice?

Fecal microbiota of recipient mice differ from donor and inoculum.

Figure 4 shows that inoculated microbiota of donor mice were distinct to recipient microbiota. The groups are separated along axis 2, but not axis 1 in multidimensional scaling (MDS) of Jaccard distances. Inoculum do not differentiate from donor microbiota, since it could not be separated by Jaccard distances. GF fecal 16S rRNA sequencing confirmed that GF mice are different from donor and recipient (separated along axis 1).

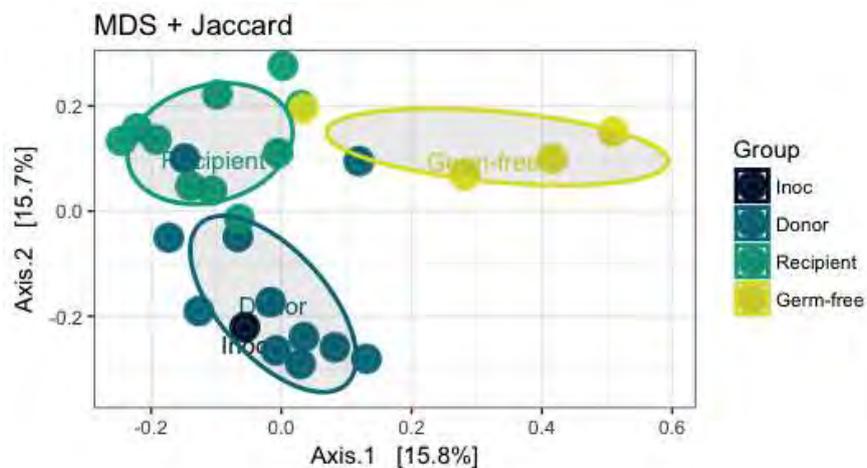


Figure 4 First two axes of a multidimensional scaling (MDS) of Jaccard distances from 16S rRNA gene sequencing of donors (conventional mice), recipients (germ-free mice colonized with the inoculum: inoc) and germ-free mice.

Microbiota colonization of GF mice is not sufficient to achieve intestinal barrier maturation observed in donor mice

Intestinal paracellular permeability to FSS is lower in donor mice compared to GF mice. (**Figure 5A**). Colonization (recipient mice) decreased slightly but not significantly intestinal paracellular permeability compared to GF mice but do not reach the same levels as donor mice. Concentration of secretory immunoglobulin A (sIgA) in feces is higher in donor mice compared to GF mice (**Figure 5B**). Colonization (recipient mice) did not significantly increase sIgA in feces compared to GF mice. Compared to donor mice, recipient mice had lower IgA. Lipocalin-2 concentration in feces is higher in donor mice compared to GF mice (**Figure 5C**). Colonization (recipient mice) did not increase lipocalin-2 concentration in feces compared to GF mice. Compared to donor mice, recipient mice had lower lipocalin 2.

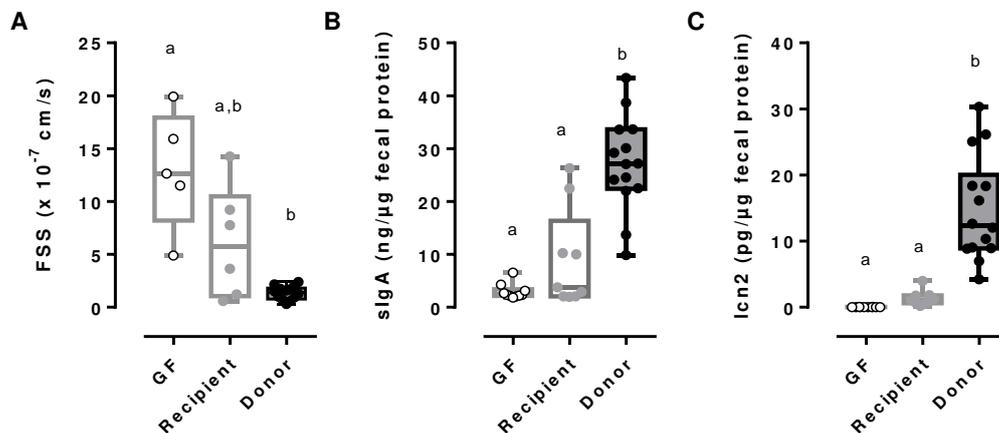


Figure 5 (A) Paracellular jejunal permeability to FSS (10^{-7} cm/s), assessed in Ussing chambers. (B) secretory IgA in fecal supernatant (sIgA) (ng/ μ g fecal protein). (C) Lipocalin-2 (lcn2) (pg/ μ g fecal protein) in fecal supernatant, Kruskal-Wallis test, same letter = no statistical difference, different letters = significant differences.

Microbiota colonization induces systemic humoral immune response

IgA and IgG concentrations in plasma were higher in donor mice compared to GF mice (**Figure 6A-B**). Colonization (recipient mice) significantly increased IgA but not IgG compared to GF mice. Compared to donor mice, recipient mice had lower IgG and IgA concentration in plasma.

Humoral response (IgA and IgG) directed against microbiota (*E.coli* lysate as a representative of bacterial luminal antigens) were higher in donor mice compared to GF mice (**Figure 6 C-D**). Colonization (recipient mice) slightly but not significantly increased anti-*E. coli* IgG and IgA compared to GF mice. Compared to donor mice, recipient mice had decreased anti-*E. coli* IgG and IgA in plasma.

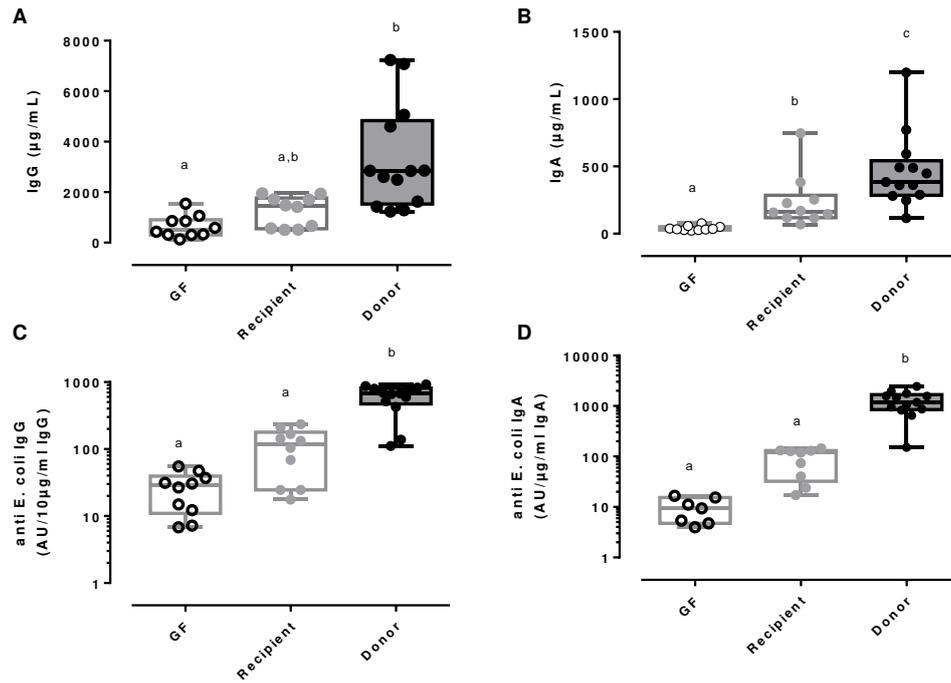


Figure 6 Plasmatic humoral immune response. **A** IgG ($\mu\text{g/mL}$). **B** IgA ($\mu\text{g/mL}$). **C** anti-*E. coli* IgG (AU/10 $\mu\text{g/mL}$ IgG). **D** anti-*E. coli* IgA (AU/ $\mu\text{g/mL}$ IgA). Kruskal-Wallis test., same letter = no statistical difference, different letters = significant differences.

Microbiota colonization triggers IL-17 secretion in response to TcR stimulation in siLP

IL-17 secretion by siLP cells in response to TcR stimulation is higher in donor mice compared to GF mice (**Figure 7A**). Similar secretions of IL-22 and IFN γ by siLP cells in response to TcR stimulation were observed between donor and GF mice (**Figure 7B-C**). Colonization (recipient mice) significantly increased IL-17 secretion in response to TcR stimulation in siLP compared to GF. Compared to donor mice, recipient mice had identical IL-17 secretion in response to TcR stimulation in siLP.

There were no differences for IL-17 (**Figure 7A**), IL-22 (**Figure 7B**) and IFN γ (**Figure 7C**) secretion in basal state between the three groups.

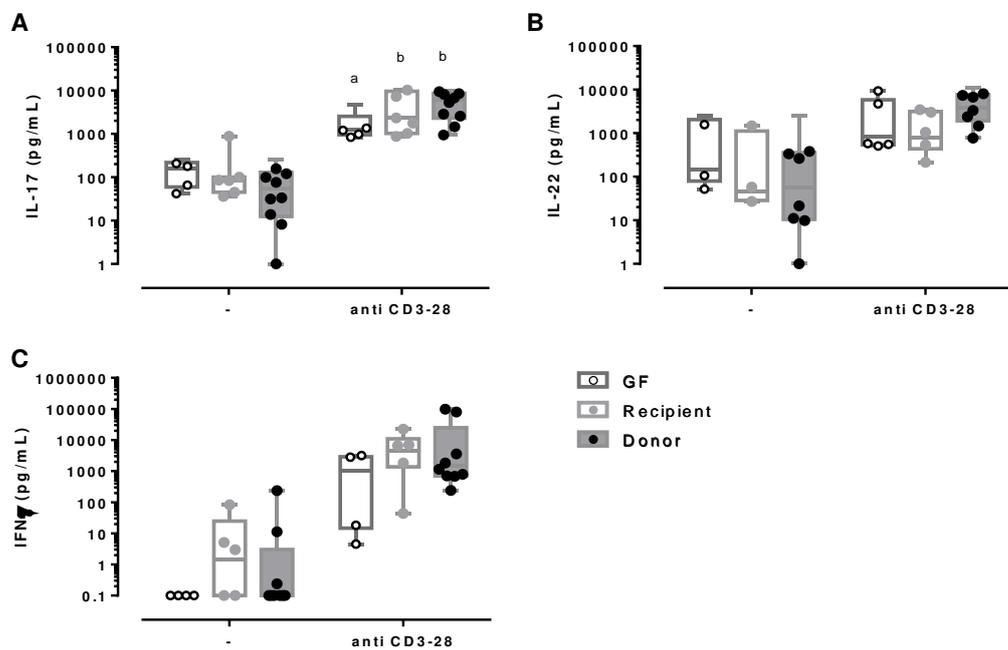


Figure 7 Primary cell culture of small intestine *lamina propria* (siLP). Cytokine concentrations in cell culture supernatant (pg/mL) after 72h incubation without or with anti-CD3/CD28 stimulation. (A) IL-17. (B) IL-22. (C) IFN γ . Two-way ANOVA, same letter = no statistical difference, different letter = significant differences.

Microbiota colonization triggers IL-17 secretion in response to TcR stimulation in spleen

On systemic level, IL-17 secretion by splenocytes in response to TcR stimulation is higher in donor mice compared to GF mice (**Figure 8A**). Similar secretions of IL-22 and IFN γ by splenocytes in response to TcR stimulation were observed between donor and GF mice (**Figure 8B-C**). Colonization (recipient mice) significantly increased IL-17 secretion in response to TcR stimulation in splenocytes compared to GF. However, recipient mice did not reach the same IL-17 concentrations than donor, so recipient mice had lower IL17 secretion in response to TcR stimulation in spleen compared to donor mice

In basal conditions, IL-22 secretion is lower in donor compared to GF and recipient mice (**Figure 8B**).

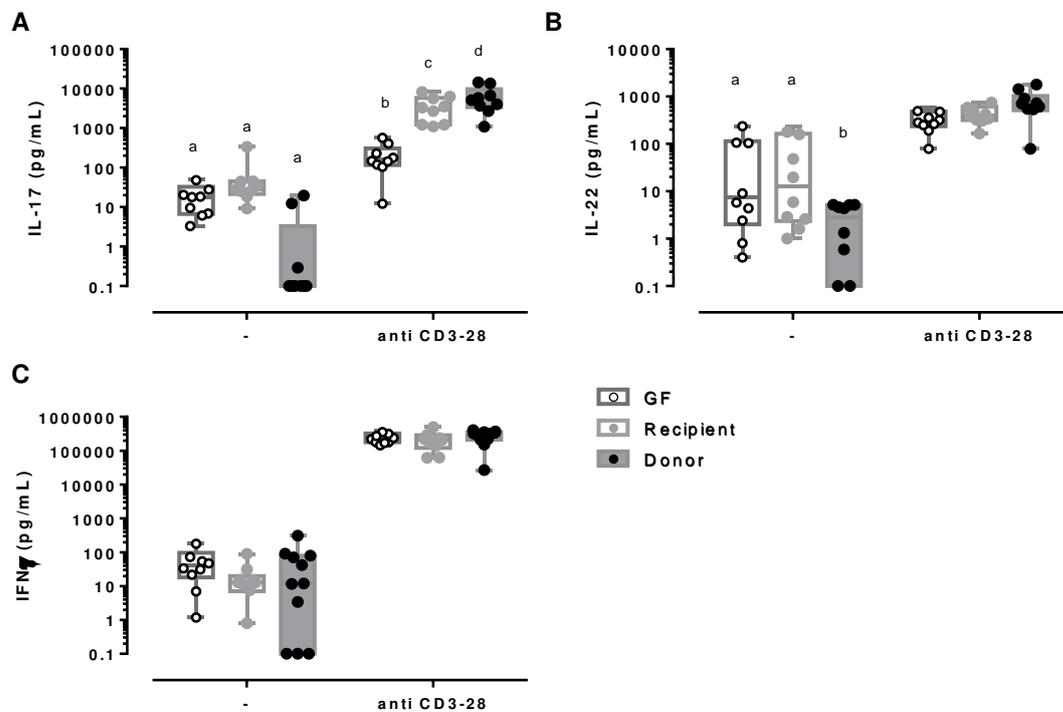


Figure 8 Primary cell culture of splenocytes. Cytokine concentrations in cell culture supernatant (pg/mL) after 72h incubation without or with *E.coli* lysate or anti-CD3/CD28 stimulation. (A) IL-17. (B) IL-22. (C) IFN γ . Two-way ANOVA, same letter = no statistical difference, different letters = significant differences.

Microbiota colonization induced glucose intolerance compared to GF and donor

Body weight was higher in donor mice compared to GF and recipient (Figure 9A). Interestingly, fasted blood glucose was lower in donor mice compared to GF and recipient mice (Figure 9B). Glucose tolerance during OGTT was better in donor and GF mice compared to recipient mice as observed through the kinetic and AUC (Figure 9C-D). Indeed, 16 weeks colonization induced glucose intolerance in recipient mice compared to GF and donor.

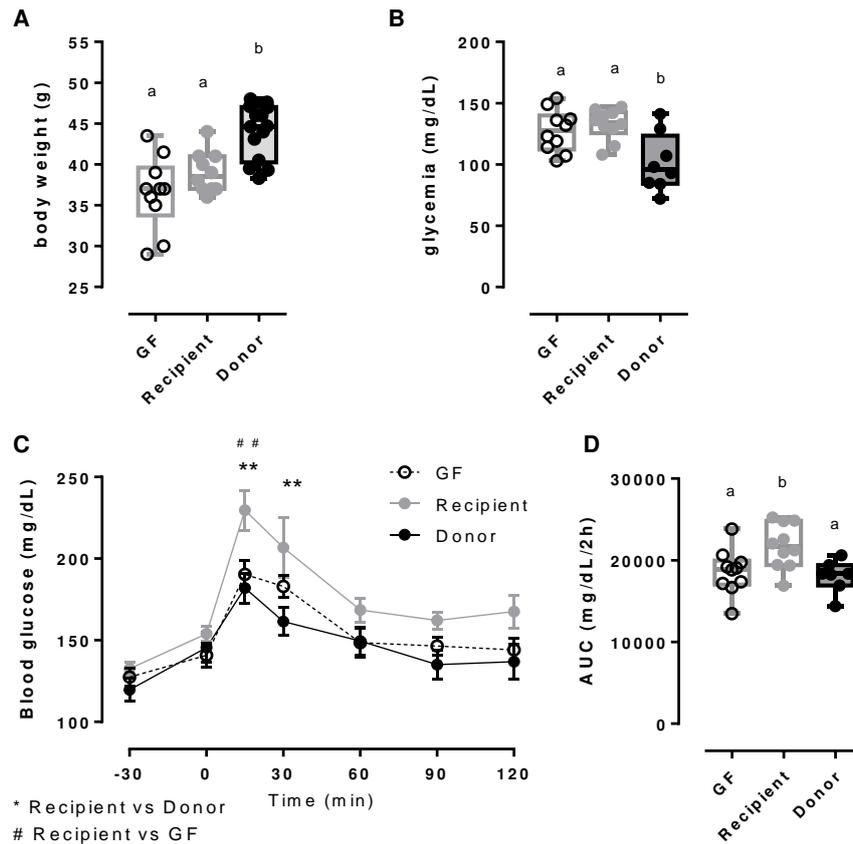


Figure 9 (A) Body weight (g). (B) 16h-fasted blood glucose (mg/dL). (C) Oral glucose tolerance test in 16h-fasted mice, blood glucose (mg/dL), two-way ANOVA, ** $p < 0.01$ recipient vs donor, ## $p < 0.01$ recipient vs GF. (D) Area under the curve (AUC) (mg/dL/2h), one-way ANOVA, same letter = no statistical difference, different letters = significant differences.

DISCUSSION

In this study, we aimed to address the role of microbiota in MS-induced glucose intolerance and associated defect of IL-17 secretion in siLP and spleen (Ilchmann-Diounou et al., 2019). We performed fecal microbiota transfer (FMT) from maternal separated (MS) and control at PND350 into germ-free (GF) adult mice and demonstrated that 16 weeks after FMT, MS microbiota did not induce glucose intolerance nor IL-17 secretion defect compared to control microbiota even though microbiota was different between mice receiving microbiota inoculum from control or MS mice.

Our experiment showed that microbiota was not sufficient to induce the MS PND350 phenotype. Microbiota dysbiosis was observed at earlier time point in the MS model (PND50) and was associated with intestinal barrier modification (lower anti-microbial peptides, intestinal hyperpermeability, low grade inflammation) without disturbed glucose metabolic phenotype (Riba et al., 2018). At PND350 in MS mice, antimicrobial peptides and intestinal permeability returned to the control level and only intestinal inflammation and microbiota dysbiosis remained. Microbiota is evolving in the course of time in the MS model (*cf.* III. Part Results. First Result, Additional Data). Therefore, we wonder if the microbiota at PND350 could be a consequence of earlier defect and if the causative microbiota could be the dysbiotic microbiota observed in MS mice at PND50. The next step will be to perform FMT with microbiota of MS mice at PND50. If microbiota from MS PND50 mice fails to induce glucose intolerance associated with decrease of IL-17 secretion in siLP and spleen it would be interesting to combine dysbiotic microbiota and impaired intestinal barrier i.e. transfer microbiota from control and MS mice on GF mice submitted or not to MS. Indeed, a weakened intestinal barrier associated with low-grade inflammation may be necessary to enable dysbiotic MS microbiota to modify host immune-metabolism. Indeed, de Palma et al. showed that microbiota transfer was not sufficient to induce MS-caused behavioral modifications in GF mice (De Palma et al., 2015). This experiment will also be an opportunity to address the consequences of MS on intestinal barrier and immune response in GF conditions. Indeed, MS has been widely used in rodent to address the long lasting consequences of early life stress on intestinal homeostasis (at PND50 irritable bowel syndrome model) but the contribution of microbiota in those outcomes has never been tested.

Even though we could not reach our primary objective, we took advantage of this work and analyzed the effect of FMT on adult GF mice in order to compare intestinal physiology as well as systemic immune response and glucose metabolism between donor, recipient and GF mice.

With our work, we showed that, 16 weeks after colonization, recipient mice failed to reach the same level of maturation that donor mice, regarding intestinal barrier, immune response and metabolic status.

First, microbiota from recipient mice is different from donor microbiota, even if inoculum was a good representative of donor microbiota. This result could be explained by the conservation of the inoculum or the frequency of the inoculum administration. FMT could be performed by co-housing, frozen inoculum without prior preparation or frozen inoculum with cryoprotectant (milk in our case). Inoculation could be performed twice a week for several weeks or in one shot, what we did. Further analysis of the bacteria involved in the distinction between donor and recipient are needed to provide explanations and also to reconcile the data on microbiota and consequence of colonization on host maturation.

Adult GF mice present defect in immune response, social behavior, nutrient absorption and metabolism (Desbonnet et al., 2014).

Indeed, in a second part, we showed that colonization only induced partial maturation of host functions. We demonstrated that intestinal permeability is lower in donor mice compared to GF and recipient mice are in-between. Data on intestinal permeability in GF mice are conflicting. An increase of transcellular intestinal permeability to HRP has been described (Heyman et al., 1986) in conventionally raised mice compared to GF. Thevaranjan et al. report no modifications of *in vivo* measured intestinal permeability in GF vs conventionally raised or colonized mice (Thevaranjan et al., 2017). It is interesting to note that colonization decreased permeability of another barrier: the Blood Brain Barrier (Braniste et al., 2014). We then looked at IgA and lipocalin production by the intestine. Donor mice have higher concentration of sIgA and lipocalin compared to GF and recipient mice. It has already been published that GF mice have weakened intestinal barrier, namely immature immune system (Falk et al., 1998; Macpherson and Harris, 2004) and impair sIgA secretion (Moreau et al., 1978). However, it has been demonstrated that colonization of GF with complex microbiota induced the same IgA response that the one observed in conventionally raised mice (Lécuyer et al., 2014). The discrepancy between those results and our results could be explain by the method used to measured IgA,

elispot in siLP (Lécuyer et al., 2014) and ELISA on feces for us. Furthermore, inoculum was prepared out of fresh microbiota and gavaged twice. They analyzed mice 8 weeks after colonization whereas we analyzed them 16 weeks after colonization. In a recent article Le Roy et al. demonstrated that stabilization of microbiota is reached 9 weeks after colonization and as such host parameters could be unstable before (Le roy front in microbial 2019). Regarding cellular intestinal immune response, GF mice are described to have thinner siLP (Round and Mazmanian, 2009) and a great deficit in gut associated lymphoid tissue which can be restored by colonization (Falk et al., 1998; Macpherson and Harris, 2004). In our study, donor produced higher IL-17 in response to TcR stimulation compared to GF and colonization completed restore IL-17 secretion in recipient mice. Indeed, it has been well described that GF mice have decreased Th17 in small intestine which can be stimulated by colonization and more particularly by Segmented Filamentous Bacteria (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2008). Interestingly, regarding IL-17 response, the same observation has been made at systemic level (spleen).

Donor mice had higher Ig in plasma compared to GF and colonization slightly increased IgA response but not IgG. Humoral response toward microbiota was higher in donor mice compared to GF and colonization failed to induce such response.

GF mice body weight is lower compared to donor mice and colonization failed to increase GF body weight so recipient mice are lighter compared to donor mice even after 16 weeks of colonization. It has been published that GF mice are lighter than conventionally raised mice (Thevaranjan et al., 2017) but colonization has been published to increase adipose mass (Bäckhed et al., 2004). Colonization induced glucose intolerance in recipient mice compared to GF and donor mice. However, it has been described that GF have better glucose tolerance than specific pathogen free (SPF) mice (Wostmann et al., 1983) but in our study we did not observed any difference between GF and donor mice regarding glucose tolerance. Microbiota colonization induces both inflammation and glucose intolerance in a dynamic pathway. Indeed, Molinaro et al. showed that colonization induced early transient inflammation associated with glucose intolerance and after 4 weeks the inflammation disappear but glucose intolerance remained (Molinaro et al., 2017). However, in this study conventionally raised mice were glucose intolerant compared to GF mice whereas we did not observed any differences in our model. This result is surprising, as aging is a major risk factor for the development of T2D, in both human and animal studies (Almaça et al., 2014; Meneilly and Tessier, 2001). Their protection from metabolic disorders are principally due to increased fatty acid metabolism,

caused by increased AMP-activated protein kinase (AMPK, a key regulator of metabolism) activity and elevated levels of Fiaf (fasting-induced adipose factor, a regulator of fatty acid metabolism) (Bäckhed et al., 2007). Beside the already mentioned difference in protocol used for inoculation of microbiota. The age of the receiver mice is of importance as well as the age of the microbiota from the donor mice.

Microbiota of PND350 mice were transferred in GF mice aged of PND150 that might be too old. There is more and more evidence for a window of opportunity in early life (Dupont et al., 2016; Ménard et al., 2008; Renz et al., 2017). The first days of life are a highly dynamic period in the development of intestinal barrier (Stockinger et al., 2011). Age of recipient is important for the establishment of transferred microbiota. Indeed, experiments of microbiota transfer into GF and SPF of different age, showed that microbiota of mice, which has been transplanted at the age of three weeks (GF or SPF) clustered more closely with inoculum than those which were 8-weeks old at transplantation (Le Roy et al., 2019). Furthermore, it has been demonstrated that colonizing with old microbiota do not lead to the same outcome than colonizing with young microbiota (Thevaranjan et al., 2017) May be the differences in our model are at least partially explained by the use of old microbiota used to colonize old GF mice. Additionally, age is also a crucial factor for immune priming. Al Nabhani et al. identified a unique time window where a so-called “weaning reaction” takes place. Indeed, microbiota changes in the timeframe of weaning provoke robust but transient immune reaction, which is crucial for later protection to immune-related pathologies (Al Nabhani et al., 2019).

In summary, this study gave us the opportunity to observe the effect of late FMT in adultGF mice on intestinal barrier and immune system maturation. This study gives interesting perspectives to better understand the role of microbiota in maturing the host, the importance of microbiota used to colonize and the time of colonization. It also provides information relative to the close relationship and regulation between intestinal barrier, immune response and glucose metabolism.

PERSPECTIVES

The established collaborations gave us the opportunity to continue the project in order to question the role of microbiota in the MS model. In ongoing experiments, we apply MS in GF mice, studying intestinal barrier. We wonder, what are the effects of MS in GF conditions.

Furthermore, we are planning to proceed to FMT experiments, inoculating MS or control microbiota in young GF mice, which underwent or not MS prior to FMT. We hope that these experiments put light on the role of microbiota in the MS phenotype.

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THIRD RESULT:
**EARLY LIFE STRESS INDUCES GLUCOSE INTOLERANCE AND
INSULIN SECRETION FAILURE IN AGING FEMALE MICE**

Work in progress

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As a third part of my PhD project, we were interested in the long-term effects of neonatal maternal separation (MS) on the female litter. We chose to analyze and present female data separately from male data, since we observed from early time point a sexual dimorphism in our model. Indeed, female and male data in early adulthood (PND50) were published in two different articles due to sexual dimorphism. Indeed, in male mice MS increased intestinal permeability and led to intestinal low-grade inflammation (Riba et al., 2018), whereas in female mice, MS induced, intestinal *E. coli* overgrowth, - (Riba et al., 2017) . Nevertheless, some observations were similar between male and female mice that underwent MS, as for example intestinal hypersensitivity, diminished intestinal lysozyme activity and elevated humoral immune response against microbiota.

We wondered what are the long-term consequences of MS in female aging mice on allostatic load, namely intestinal barrier function, neuroendocrine functions and metabolism. Additionally, we were interested in immune response and wondered if MS could lead to autoimmune disorder in aging female mice. As previously discussed in the review article, there seems to be a link between stressful environment, intestinal barrier dysfunction and autoimmune disorders.

This work is still ongoing within the framework of a collaboration with Prof Jamileh Movassat, Biology and Pathology of the Endocrine Pancreas at BFA, Université Paris Diderot, Paris and in collaboration with Dr Julien Diana, Innate and Adaptive Immune Pathways in Autoimmunity and Autoinflammation at INEM, Inserm, Paris.

The project was supported by the European Society of Pediatric Endocrinology (ESPE), which financed my two-month stay (December 2017 – January 2018) in the laboratory of Prof Jamileh Movassat.

WORK IN PROGRESS

**EARLY LIFE STRESS INDUCES GLUCOSE INTOLERANCE AND INSULIN
SECRETION FAILURE IN AGING FEMALE MICE**

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KEY WORDS

Intestinal barrier, Intestinal permeability, DOHaD, Non-communicable diseases, Type 1 diabetes

ABSTRACT

The incidence of metabolic disorders is increasing worldwide. Besides diet and life style habits, epidemiological studies highlighted an association between stress and the increase incidence of non-communicable diseases (NCD) including metabolic disorders, inflammatory and autoimmune diseases. Based on the concept of Developmental Origins of Health and Diseases (DOHaD) our study aimed to investigate whether early life stress can trigger NCD and associated key feature i.e. low-grade inflammation.

Maternal separation (MS) is an established model of early life stress in rodent. C3H/HeN mice pups were separated from their dam and the rest of the litter 3 hours per day during 10 days starting at post-natal day 2 (PND2). All experiments were carried out in female offspring aged of PND350 on standard diet.

MS had an impact on intestinal immune system at PND350 but not on intestinal permeability. Secretion of IL-17 and IL-22 by small intestine *Lamina Propria* (siLP) cells in response to Tcell receptor (TcR) stimulation was increased by MS as well as TNF α secretion with and without LPS stimulation. Additionally, MS led to low-grade inflammation: IFN γ -secretion by splenocytes in response to TcR stimulation was increased. MS decreased body weight and induced glucose intolerance, measured during oral glucose tolerance test (OGTT). MS did not induce a loss of insulin sensitivity measured by intraperitoneal insulin tolerance test (ITT). Instead, MS decreased plasma insulin secretion in response to glucose stimulation. Furthermore, ratio of β -cell surface to pancreas surface was slightly decreased in MS mice and this ratio positively correlates with insulin secretion induced by glucose.

For the first time, this study showed that early life stress induces glucose intolerance associated with a loss of insulin secretion in mice non-genetically predisposed to metabolic disorders or type 1 diabetes and fed with standard diet. Interestingly, glucose intolerance is associated with local and systemic low-grade inflammation but not with intestinal hyperpermeability.

INTRODUCTION

The early life period is crucial for the development and maturation of metabolism, immune system, intestinal barrier and a life-long beneficial host-microbiota interaction (Stockinger *et al.*, 2011). The concept of Developmental Origins of Health and Disease (DOHaD) highlights the importance of early life period and raises the hypothesis that chronic diseases could find their origins in perinatal environment (Barker *et al.*, 1989; Gluckman *et al.*, 2016). Apart of genetics and life style, early life environment could leave a silent imprinting in child's physiology, which possibly erupt later in pathology. This imprinting is also called allostatic load and usually measured through neuroendocrine, metabolic, inflammatory and cardiovascular markers (Edes & Crews, 2017).

Early life psychological stress is one of multiple factors of interest. Epidemiological studies have shown an association between early life stress and increased circulation inflammatory markers in young adults (Baldwin *et al.*, 2017). Neonatal maternal separation (MS) is a stress model widely used in rodents as a paradigm of early life adverse events. We previously observed that, in female mice, MS triggers lasting alterations of intestinal homeostasis in young adult offspring (post-natal-day (PND) 50) including defect of Paneth cells and low-grade inflammation (Riba *et al.*, 2017).

In this study, we aimed to investigate in aging wild-type mice under standard diet the long-term effects (PND350) of neonatal MS on mice's allostatic load: intestinal barrier function, low-grade inflammation, neuroendocrine functions, as well as glucose metabolism.

EXPERIMENTAL PROCEDURES

Mouse model

All experimental protocols were approved by local Animal Care Use Committee (Comité d'Ethique de Pharmacologie-Toxicologie de Toulouse - Midi-Pyrénées, France) registered as N°86 at the Ministry of Research and Higher Education (N° 0029/SMVT), and conducted in accordance with the European directive 2010/63/UE. Mice were kept at a constant temperature ($22 \pm 1^\circ\text{C}$) and maintained on a 12:12 h light/dark cycle (lights on 7h30 am) on Specific and Opportunistic Pathogen Free (SOPF) conditions. Normal diet (Harlan2018, Envigo, Gannat, France) and water were available *ad libitum*.

Maternal Separation protocol

Nulliparous female C3H/HeN mice (Janvier Labs, Le Genest St Isle, France) were used. Maternal separation (MS) was performed as previously described (7). Briefly, pups were separated from their dam and the rest of the litter 3 hours per day. MS was repeated for 10 working days, weekend excluded, between post-natal-day 2 (PND2) and PND15. Control pups were left with their dam. At weaning (PND21), litters were mixed within the same group; only females were kept for this study. Four independent batches of experiments were realized. Experiments were performed at PND350.

Oral glucose (OGTT) and intraperitoneal insulin tolerance test (ITT)

OGTT and ITT were performed in mice 6 h-fasted during day light. For OGTT, mice were gavaged with 2 mg glucose per g of bodyweight. Blood glucose levels were monitored from the tip of the tail vein with a glucose meter (Johnson & Johnson, Issy-les-Moulineaux, France) at -30, 0 (glucose gavage), 15, 30, 60, 90 and 120 min.

For plasma insulin, blood samples were harvested in fasted state (6h) and 15 min after glucose stimulation *per os* (2 mg glucose per g of bodyweight). Insulin was measured with commercial ELISA kit (Merck Millipore, Saint Quentin en Yvelines, France).

During ITT mice were injected with 0.75 mU insulin (NovoRapid, Novo Nordisk, Chartres, France) per g of bodyweight. Blood glucose levels were measured up to 30 min after injection.

For fasted blood glucose, mice were fasted 16-h overnight.

Lysozyme activity in fecal content

Activity of lysozyme against the peptidoglycan was determined in feces suspended in phosphate-buffered saline using the EnzChek Lysozyme Assay Kit (Molecular probes, Life Technology, St Aubin, France).

Intestinal permeability in Ussing chambers

Intestinal permeability was assessed as previously described (Riba *et al.*, 2018). Briefly, jejunal and colonic fragments were mounted in Ussing chambers (Physiologic Instruments, San Diego, CA, USA). Tissues were bathed 2h with oxygenated thermostated Krebs's solution (Sigma, Saint Quentin Fallavier, France). Fluorescein Sodium Salt 40 µg/ml (FSS 376 Da; Sigma) and Horse Radish Peroxidase 0.4 mg/ml (HRP 4 kDa; Sigma) were respectively added to mucosal compartment as para- and trans-cellular markers of intestinal permeability.

Intestinal permeability to total HRP was determined by ELISA. Briefly, 96-wells black plates (Greiner, Les Ulis, France) were coated with 10 µg/ml mouse polyclonal to HRP (Abcam, Paris, France), blocked with PBS-1% bovine serum albumin (BSA), incubated with serosal compartments of Ussing chamber, detected with 10µg/ml Rabbit polyclonal anti HRP biotin (Abcam) and revealed with FITC-conjugated streptavidin (BD, Paris, France). Fluorescence intensity was measured 485 nm/525 nm using an automatic Infinite M200 microplate reader (Tecan, Männedorf, Switzerland). Intestinal permeability to FSS was determined by measuring the fluorescence intensity (FI) 485 nm/525 nm using an automatic Infinite M200 microplate reader. Permeability was calculated as the ratio of flux/concentration, and expressed as cm/second.

Immune cells isolation

Splenocytes were isolated through a 70-µm nylon mesh and suspended in PBS 1%-KO SR serum (Gibco, Thermofisher Scientific).

Isolated cells from small intestines (si) *lamina propria* (siLP) were obtained as follow: si were washed in cold PBS, incubated in PBS 3 mM EDTA (Sigma), washed in warm PBS, digested with 100 U/mL of collagenase (Sigma) in DMEM 20% FCS and finally purified on Percoll (Sigma).

Fluorescence-Activated Cell Sorter Analysis

Isolated cells from spleen and siLP were stained as follow. Activated T-cells: CD4 (BD), CD44 (BD), CD62L (BD); Regulatory T-cells: CD4 (BD), CD25 (BD), Foxp3 (ebioscience,

ThermoFisher Scientific); ILC3: CD127 (BD), ROR γ t (BD). Th17/22 CD3 (BD), ROR γ t (BD). MACSQuant® Analyzers (Miltenyi Biotec SA, Paris, France) and VenturiOne® (AppliedCytometry, Sheffield, Great Britain) software were respectively used for data collection and analysis.

Primary cell culture

Isolated cells from spleen and siLP were seeded at 2×10^6 cells per well in the presence or absence of a) 100 ng/mL Lipopolysaccharide (LPS; Sigma), b) 1 μ g/mL of protein from commensal *E. coli* lysate or c) 5 μ g/mL hamster anti-mouse CD3 (BD) and hamster anti-mouse CD28 (BD) coated wells. Supernatants were collected after a) 24h, b/c) 72h.

Cytokines measurement

Cytokines were measured in supernatant of primary cell culture, or jejunal fragments suspended in RIPA buffer (0.5% deoxycholate, 0.1% SDS and 1% Igepal in TBS) containing complete anti protease cocktail (Sigma). Jejunal protein concentrations were measured using BCA uptima kit (Interchim, Montlucon, France). IL-17, TNF α , IL-10, IL-22, TGF β and IFN γ in supernatant or lysate of jejunal fragments were assayed using commercial ELISA kits (R&D Systems, Lille, France).

Humoral response in feces and plasma

Plates were coated with 5 μ g/ml of sheep anti-mouse IgA (Sigma) or goat anti-mouse IgG (SouthernBiotech, Cliniscience, Nanterre, France), incubated with plasma, detected with 1.5 μ g/ml HRP-conjugated goat anti-mouse IgA (Sigma) or goat anti-mouse IgG (SouthernBiotech), HRP was revealed using TMB and the reaction was stopped with H₂SO₄ 2N before reading at 450nm using automatic Infinite M200 microplate reader.

Measurements in plasma

ELISA kits were used to monitor corticosterone (Immunodiagnostic Systems, Pouilly-en-Auxois, France), GIP and GLP-1 (Merck Millipore, Saint Quentin en Yvelines, France). Plasma-cholesterol, LDL, HDL, triglycerides, free fatty acids and calcium were analyzed by the Platform GenoToul Anexplo, Toulouse, France.

Staining and counting of β -cells

Pancreas were fixed over night in Formol-4%, rinsed in ethanol-70% and included in paraffin. Entire pancreas were cut in 5 μ m slices, representative slices across the organ were mounted on slides, first stained with anti-insulin-guinea pig (Abcam) and anti-glucagon-rabbit

(Europroximia), second incubated with anti-guinea pig-HRP (Abcam) and anti-rabbit-HRP (Jackson), third revealed with diaminobenzidine (Vectorlabs). Surface counts were realized using Microvision HistoLab 10.5. Ratio of total insulin-positive-surface to total pancreas-surface was calculated.

Statistical analysis

Statistical analysis were performed using GraphPad Prism version 6.04 (GraphPad Software, La Jolla, CA, USA). Results for single comparisons were displayed as box plots [min to max] and analyzed using unpaired t-test or Mann-Whitney test after prior Shapiro-Wilk Normality test and F-Test to compare variances. Multiple groups were displayed either as kinetics with SEM or box plots [min to max] and compared per family by Bonferroni posttest after a significant two-way ANOVA. Differences were considered significant for $P < 0.05$.

RESULTS

MS had no effect on fecal lysozyme nor intestinal permeability.

Since maternal separation is a model of intestinal irritable bowel syndrome at PND50, we wondered if the intestinal barrier is disturbed in our model at long term. First, lysozyme activity in feces of PND350 mice was not different (Figure 1A). Second, translocation of intestinal bacterial fragments was assessed indirectly by LPS Binding Protein (LBP) concentrations in plasma, without modification in MS mice (Figure 1B). Additionally, we addressed intestinal permeability *ex-vivo* in Ussing chambers in jejunum (Figure 1C-E) and colon (data not shown). No difference for electrical resistance, para- (Fluorescein Sodium Salt, FSS) and trans-cellular permeability (Horse Radish Peroxidase, HRP) was observed between MS and control mice.

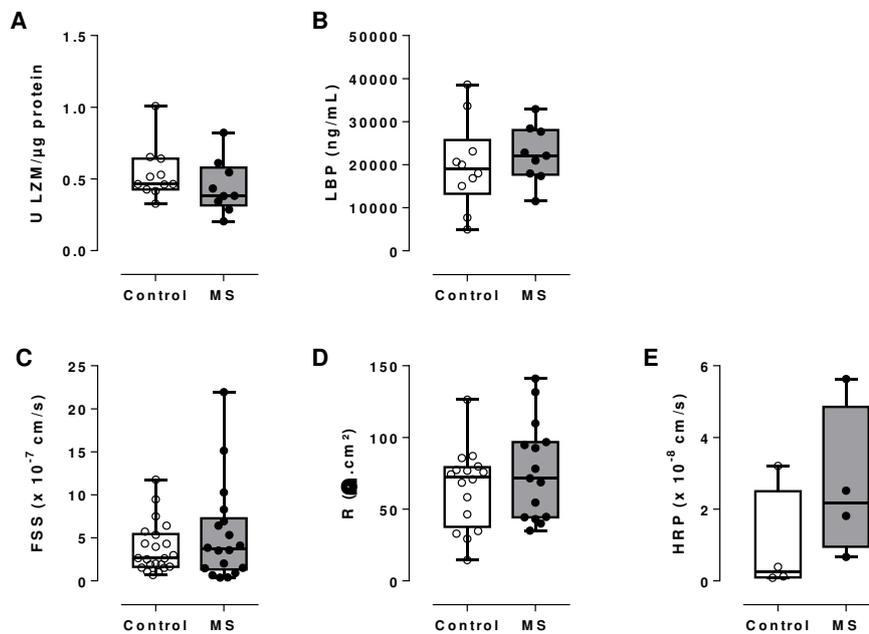


Figure 1 MS did not affect fecal lysozyme nor intestinal permeability. **(A)** Lysozyme (LYM) activity in fecal content (U LYM/ μg protein). **(B)** LPS-binding protein (LBP) in plasma (ng/mL). **(C-E)** Jejunal permeability as assessed in Ussing chambers. **(C)** Paracellular permeability of jejunum to FSS ($\times 10^{-7}$ cm/s). **(D)** Electrical resistance of jejunal tissue (Ωcm^2). **(E)** Transcellular permeability of jejunum to total HRP ($\times 10^{-8}$ cm/s).

MS increased cellular response in siLP

We analyzed intestinal immune response. First, fecal IgG and IgA were similar between both groups (Figure 2A-B).

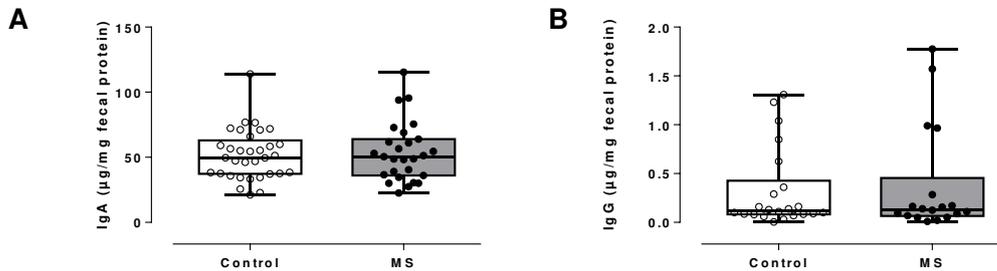


Figure 2 MS had no effect on fecal Ig. (A) Fecal IgA content (µg/mg fecal protein). (B) Fecal IgG content (µg/mg fecal protein).

Second, we looked at the cellular immune response in the small intestine *lamina propria* (siLP). Percentage of Ror γ t⁺ in CD3⁺ (Th17/22 cells) was not affected by MS (Figure 3A). Innate Lymphoid Cells 3 (ILC3: CD127⁺ ROR γ t⁺) (Figure 3B) and regulatory T cells (Treg: CD25⁺ Foxp3⁺ in CD4⁺) (Figure 3C) were not altered in MS mice either. TNF α -secretion in basal state and in response to LPS-stimulation was significantly increased in siLP cells from MS mice (Figure 3D). IFN γ -secretion was not modified by MS (Figure 3E). Nevertheless, IL-17 and IL-22-secretion in response to TcR-stimulation (anti CD3/CD28) was significantly increased in isolated siLP cells from MS mice (Figure 3F-G). We observed the same tendency for basal state and *E. coli* lysate stimulation. IL-10 and TGF β -secretion were not modified by MS (data not shown).

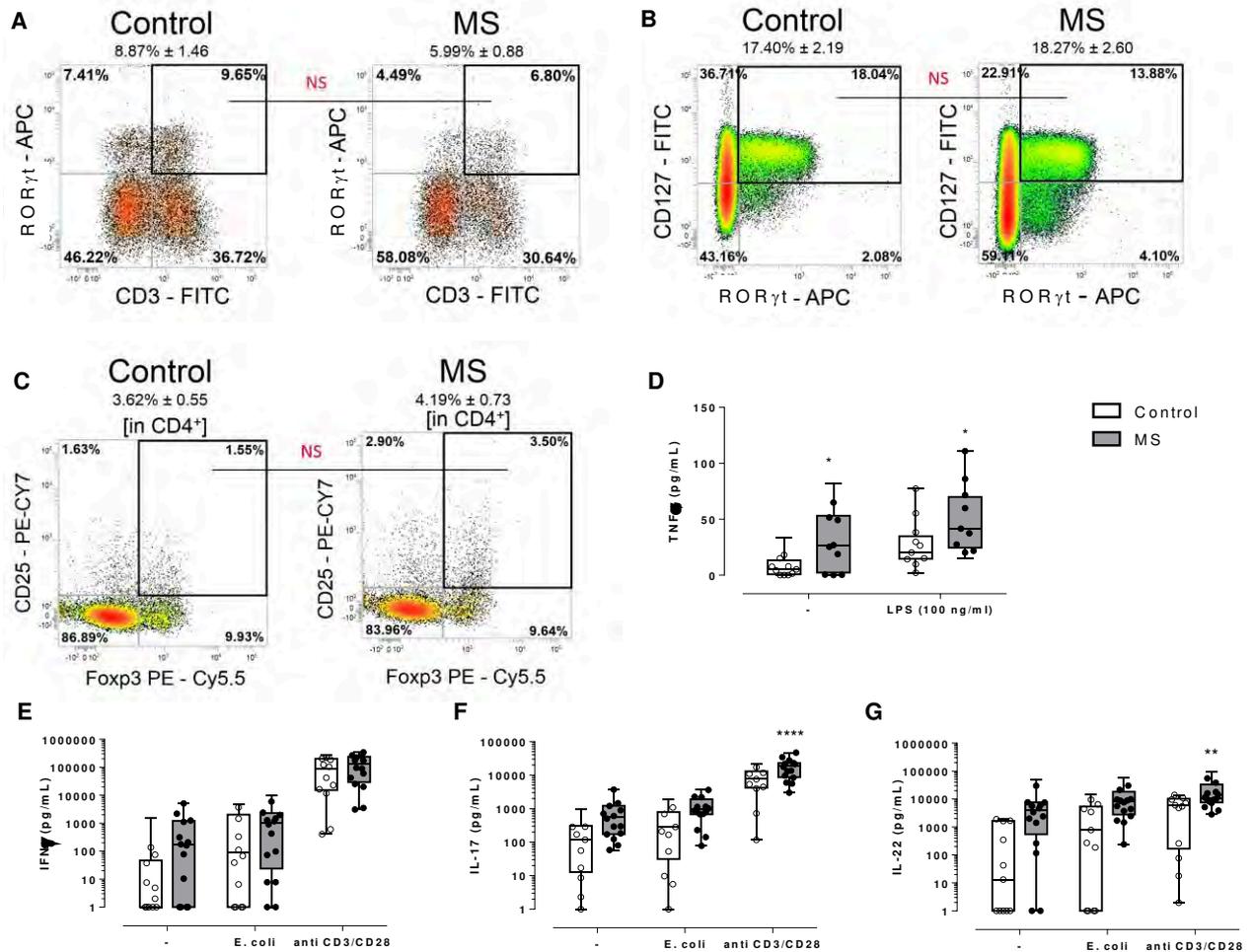


Figure 3 MS increased IL-17, IL-22 and TNF α secretions in small intestine *lamina propria* (siLP). (A) Representatives dot plots of CD3⁺ROR γ t⁺ cells of siLP. (B) ILC3 in isolated siLP (ROR γ t⁺ CD127⁺) (C) regulatory T cells in siLP (CD25⁺Foxp3⁺ in CD4⁺) (D) TNF α secretion in siLP cell culture after 24h with or without LPS (100 ng/mL) stimulation, two-way ANOVA, * p < 0.05. (E)-(G) Cytokine secretion in siLP cell culture after 72h without, with *E. coli* lysate (1 μ g/mL) or anti-CD3/CD28 (5 μ g/mL) stimulation. (E) IFN γ . (F) IL-17. (G) IL-22, two-way ANOVA, ** p < 0.01, * p < 0.0001.

MS had no consequences on humoral response in plasma

We next assessed the systemic consequences of MS. We looked at plasmatic IgG and IgA concentrations, which were similar between both groups (Figure 4A, B).

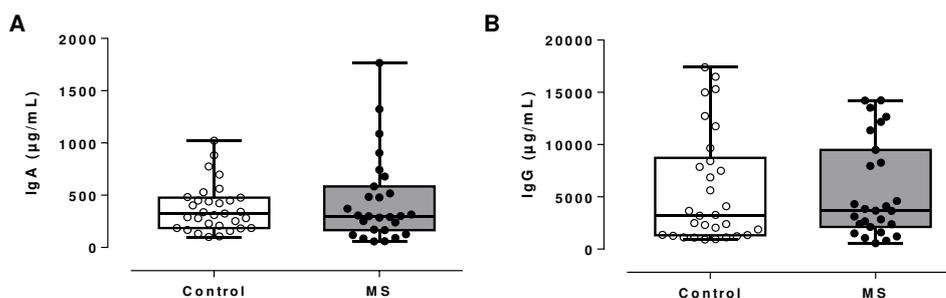


Figure 4 MS did not affect circulating IgA and IgG. (A) Plasmatic IgA (μ g/mL). (B) Plasmatic IgG (μ g/mL).

We further analyzed systemic immune response by isolation of splenocytes. Activated T cells ($CD4^+ CD44^{high} CD62L^{low}$) (Figure 5A) and regulatory T cells ($CD4^+ CD25^+ foxp3^+$) (Figure 5B) populations in spleen were not modified by MS. Regarding functionality, $TNF\alpha$ -secretion in response to LPS-stimulation was not different between both groups (Figure 5C). $IFN\gamma$ -secretion in response to TcR-stimulation was significantly higher in MS mice (Figure 5D). However, IL-17 and IL-22 secretion in response to TcR-stimulation were not modified by MS (Figure 5E, F).

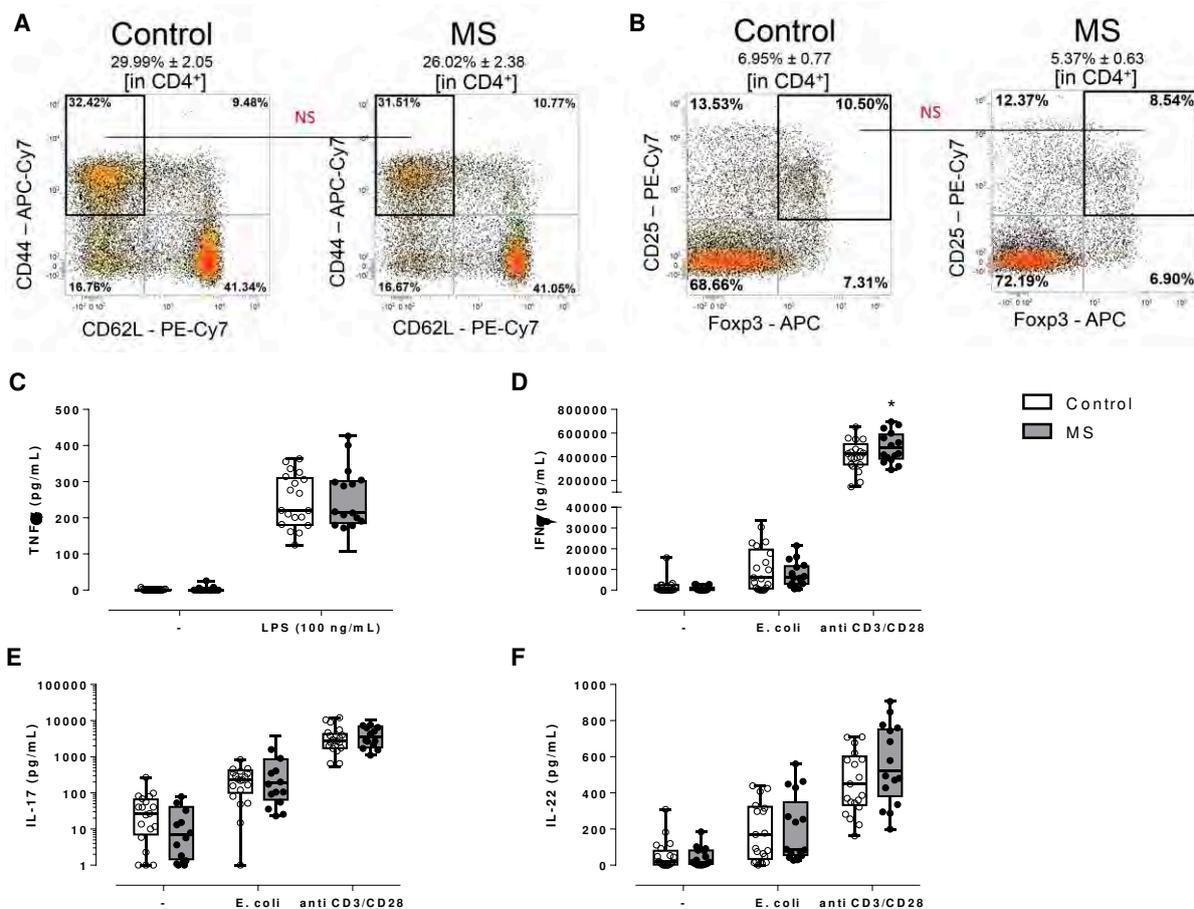


Figure 5 MS increased $IFN\gamma$ -secretion in spleen. (A) Representative dot plots of activated T cell population in spleen ($CD44^{high} CD62L^{low}$ in $CD4^+$). (B) Representative dot plots of regulatory T cell population in spleen ($CD25^+ Foxp3^+$ in $CD4^+$). (C) $TNF\alpha$ secretion in primary cell culture of splenocytes after 24h with or without LPS (100 ng/mL) stimulation (D-F) Cytokine secretion in primary cell culture of splenocytes after 72h without, with *E. coli* lysate (1 μ g/mL) or anti-CD3/CD28 (5 μ g/mL) stimulation. (D) $IFN\gamma$. (E) IL-17. (F) IL-22, two-way ANOVA, * $p < 0.05$.

MS impaired glucose tolerance associated with a decrease of insulin secretion

Regarding glucose metabolism phenotype, at PND350, MS mice had slightly but significantly lower body weight than control mice (Figure 6A) without modification of food intake (data not shown). During oral glucose tolerance test (OGTT), blood glucose levels were

higher in MS mice than in control (Figure 6B) resulting in significantly higher Area Under the Curve (AUC) for MS mice (Figure 6C). Insulin tolerance test (ITT) was not different between groups (Figure 6D, E). Insulin secretion after glucose stimulation (+15 min) justified for bodyweight was significantly decreased in MS mice compared to control (Figure 6F).

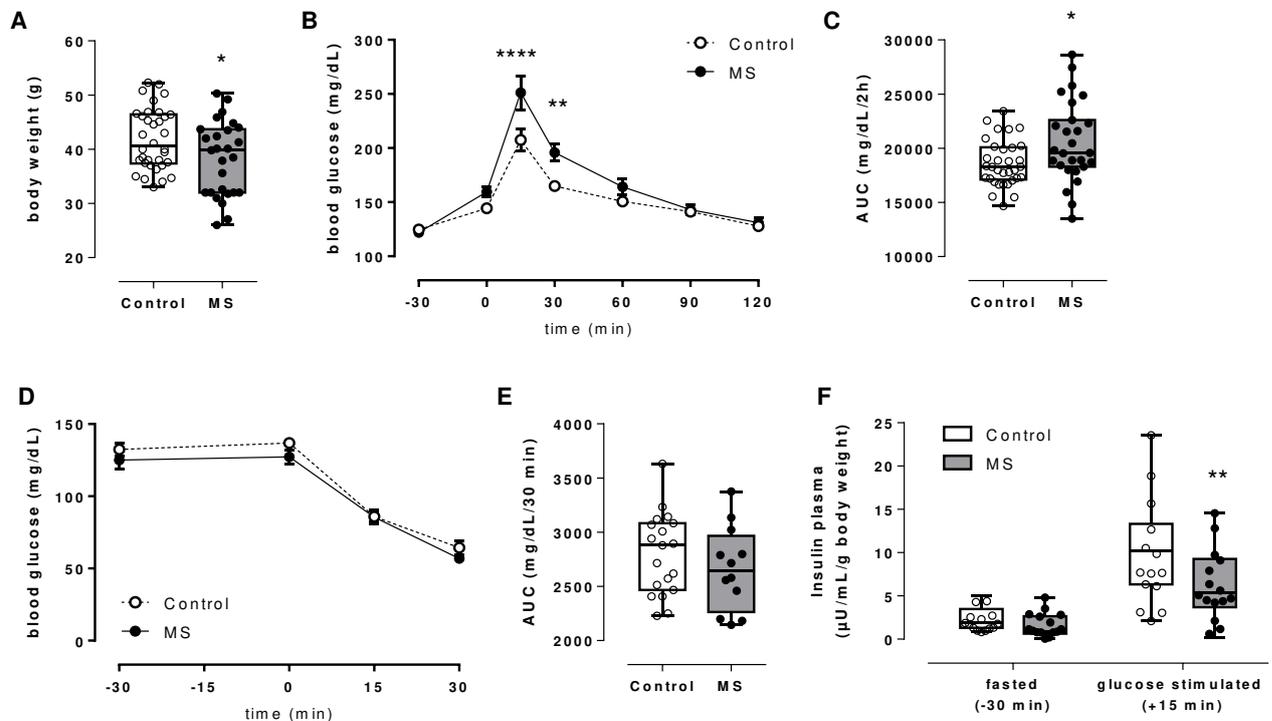


Figure 6 MS induced oral glucose intolerance associated with a loss of insulin secretion. (A) Body weight (g), unpaired t-test, * $p < 0.05$. (B) Oral glucose tolerance test, after 6-h fasting, gavage with 2 mg glucose/g bodyweight at 0, blood glucose (mg/dL) from -30 min to 120 min, two-way-ANOVA, ** $p < 0.01$, **** $p < 0.0001$. (C) Area under the curve (AUC) of blood glucose 0-120 min (mg/dL/2h), unpaired t-test. (D) Insulin tolerance test, after 6-h fasting, intraperitoneal injection of 0.75 mU insulin/g bodyweight at 0, blood glucose (mg/dL) from -30 min to 30 min. (E) Area under the curve of blood glucose 0-30 min (mg/dL/30 min). (F) Insulin secretion in 6h-fasted state and after glucose stimulation (2 mg glucose/g bodyweight) justified for body weight, two-way-ANOVA, ** $p < 0.01$.

MS did not affect plasmatic marker of stress or metabolism

No differences in plasmatic markers of metabolism like cholesterol, HDL, LDL, triglycerides or free fatty acids were observed (Figure 7A-E). There were no differences in plasma corticosterone (Figure 7F) and incretins (GIP, GLP-1, Figure 7G-H).

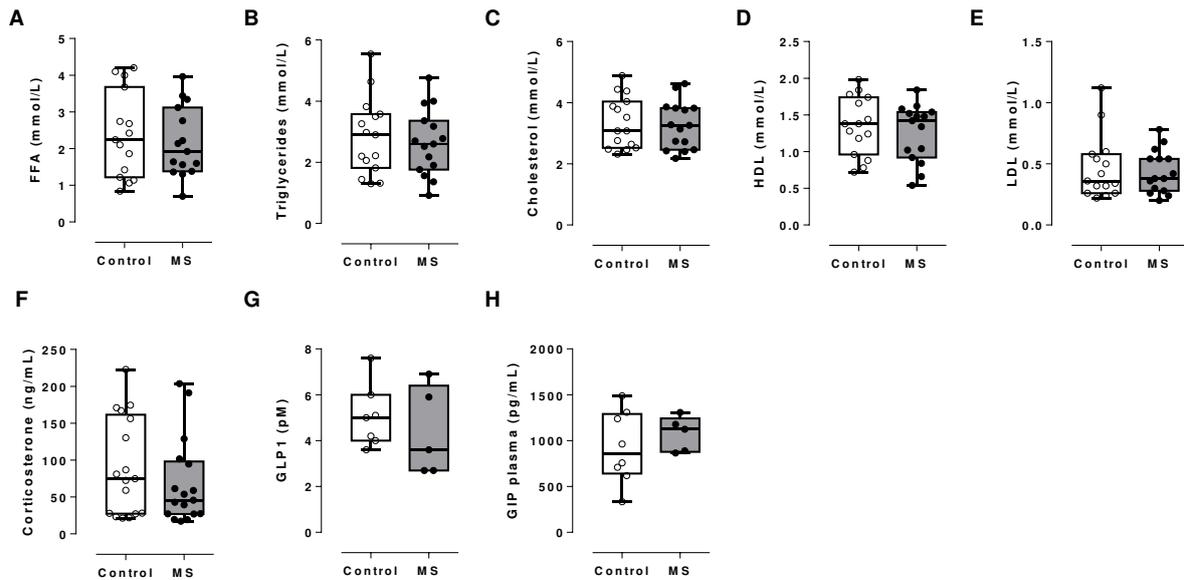


Figure 7 MS did not induce changes in metabolic plasmatic markers. (A) Free fatty acids (FFA) (mmol/L). **(B)** Triglycerides (mmol/L). **(C)** Cholesterol (mmol/L). **(D)** High-density lipoprotein (HDL) (mmol/L). **(E)** Low-density lipoprotein (LDL) (mmol/L). **(F)** Corticosterone (ng/mL). **(G)** Glucagon like peptide-1 (GLP-1) (pM). **(H)** Glucose insulinotropic peptide (GIP) (pg/mL).

MS decreased β -cell surface in pancreas

Because of the observed decrease of insulin secretion in response to glucose stimulation, we wondered if in our model pancreas β -cells producing insulin were affected. We assessed β -cell surface in pancreas by immunohistochemistry. The ratio of β -cell surface to total pancreas surface was slightly but not significantly decreased for MS mice compared to control (Figure 8A). We wondered if we could find a correlation between β -cell surface/ pancreas surface and insulin secretion in response to glucose stimulation. Indeed, these two factors are positively correlated and values of MS mice tend to be inferior to control (Figure 8B).

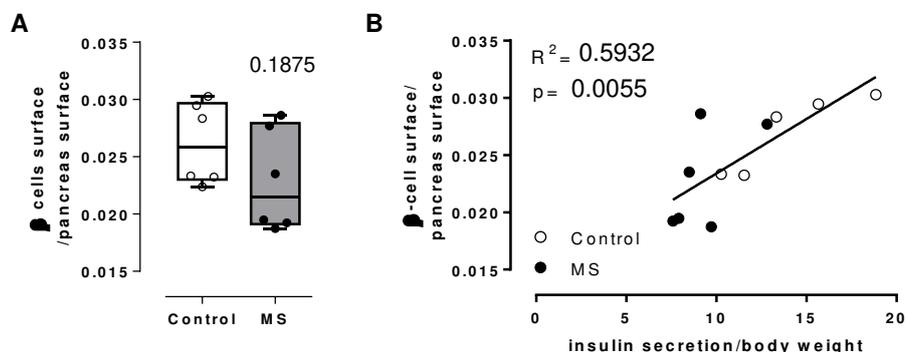


Figure 8 MS leads to diminished β -cell surface. (A) total β -cell surface/total pancreas surface **(B)** positive correlation between β -cell surface/pancreas surface and insulin secretion/bodyweight.

DISCUSSION

We here show that maternal separation (MS) has a long-lasting impact on allostatic load. MS exacerbates IL-17 and IL-22 intestinal immune response as well as intestinal and systemic low-grade inflammation at PND350 in C3H/HeN female mice. MS leads also to glucose intolerance associated with a lower stimulation of insulin secretion by glucose and a slight decrease of ratio β -cell surface / pancreas surface. These results mimic type 1 diabetes features even though we are in non-genetically predisposed mice.

As previously shown, at PND50 MS induces intestinal barrier dysfunction: defect of Paneth cells and intestinal low-grade inflammation (Riba *et al.*, 2017). Interestingly, at PND350 we do not observe anymore the decrease of fecal lysozyme activity but intestinal low-grade inflammation is persisting. Intestinal permeability was never affected in female by MS (PND50 nor PND350). In our model of MS at PND350 in female, MS induced glucose intolerance and defect of insulin secretion associated with increased intestinal and systemic IL-17 secretion in response to TcR stimulation. Furthermore, IL22 secretion in response to TcR stimulation was also increased in siLP of MS mice. The increased secretion of IL-17 and IL-22 is not due to modification of populations, but rather to a hyperfunction of Th17 and Th22. Interestingly, Th22 and Th17 levels were significantly elevated in patients of T1D (Xu *et al.*, 2014).

Stress hormones (glucocorticoids) known to regulate metabolism (Schäcke *et al.*, 2002) and immune function (Cain & Cidlowski, 2017) like corticosterone are not affected by MS at PND350 excluding a direct effect of corticosterone on the observed metabolic and immune phenotype.

Interestingly, in our study, the MS-induced glucose intolerance and loss of insulin secretion were associated with slight but significantly lower body weight. Neither plasmatic markers mainly involved in metabolic disorders (FFA, triglycerides, cholesterol, HDL, LDL) nor incretins, like GLP-1 and GIP, were modified by MS at PND350.

Pancreas is immature at birth and weaning is necessary for the maturation of β cells producing insulin (Stolovich-Rain *et al.*, 2015). Early life period is as such a critical window for pancreas maturation and scattering evidences suggest that stress might impair appropriate pancreas maturation. There is a slight correlation between bereavement and type 1 diabetes incidence after 11 years of age (Virk *et al.*, 2015). β -cell mass is influenced by early life environment. Adverse nutritional status *in utero* and early life can impact β -cell development (Portha *et al.*, 2011).

Taking together the results of this study show that MS in wild type mice under normal diet leaves a long-lasting imprinting on allostatic load. MS induces glucose intolerance associated with a loss of insulin secretion in response to glucose stimulation. Finally, this study highlights early life stress as a risk factor for glucose metabolism development independently of nutritional challenge and the early life period as critical time window for establishment of appropriate establishment of immune system and metabolism.

PERSPECTIVES

This work is still in progress and two aspects are under investigation:

1. Diabetes incidence in NOD/ShiltJ mice

We aim to perform MS in mice genetically prone to type 1 diabetes (T1D): NOD/ShiltJ mice and identify MS as a potential risk factor for autoimmune T1D. This perspective is of particular interest, since it will strengthen the hypothesis defended in our review presented in the introduction section “Psychological Stress, Intestinal Barrier Dysfunctions and Autoimmune Disorders”

Male mice, which are more resistant to T1D development, will be kept to be able to appreciate both incidence and gravity of diabetes with and without MS. This experiment will be performed within the framework of a collaboration with Dr Julien Diana, INSERM, Institut Necker Enfant Malades, Paris.

2. Role of CRAMP antimicrobial peptide in diabetes triggered by MS.

Antimicrobial peptides are also playing a role in the development of diabetes in non-obese diabetic mice model. CRAMP an antimicrobial peptides expressed in pancreas islet cells is protective for development of type 1 diabetes in NOD mice (Sun *et al.*, 2015). Interestingly, CRAMP is also expressed in small intestine of mice but only from birth to PND15 (Ménard *et al.*, 2008), corresponding to the period of MS stress. CRAMP will be stained in the pancreas of C3H/HeN mice aged of PND350 submitted or not to MS and in intestine of NOD mice submitted or not to MS and aged of PND7 and PND15. This work will also be performed within the framework of a collaboration with Dr Julien Diana, INSERM, Institut Necker Enfant Malades, Paris.

ACKNOWLEDGEMENTS

The authors declare they have no actual or potential competing financial interests.

This work was partially financed by the European Society for Pediatric Endocrinology by the Early Career Scientific Development Grant for Hanna Ilchmann.

We thank the platform GenoToul Anexplo, Toulouse, France for plasma analysis, M2C, Toxalim, Toulouse, France for assistance in cytometry analysis. Maithé Taubert, Childrens Hospital Purpan, Toulouse, France for scientific advice.

Hanna Ilchmann is a recipient of French Ministry of Research Grant.

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**FOURTH RESULT:
NON-OBESE DIABETIC MOUSE MODEL IS EMPHASIZING
DIVERGENCE BETWEEN *IN VIVO* AND *EX VIVO* INTESTINAL
PERMEABILITY MEASUREMENTS**

Manuscript submitted 23 July 2019 to Scientific Reports

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Vassilia; Denis, Colette; Ménard, Sandrine

A fourth part of my PhD project had a methodological orientation. There are various methods to evaluate intestinal permeability. We set up a project to compare *in vivo* and *ex vivo* measurements in a model of autoimmune type 1 diabetes, the NOD/ShiltJ mouse strain.

Our aim is to initiate a discussion around the definitions of intestinal permeability and systemic exposure in order to clarify the concept of intestinal permeability.

This work has been realized in collaboration with Dr Colette Denis and Dr Marie Buléon from Renal Fibrosis Lab, I2MC, Inserm, Toulouse.

Non-Obese Diabetic Mouse Model is emphasizing divergence between *in vivo* and *ex vivo* intestinal permeability measurements

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ABSTRACT

Intestinal permeability (IP) is the ability of luminal antigens to pass intestinal epithelium. We aimed to compare IP measured by *in vivo* and *ex vivo* technics in a mouse model of type 1 diabetes mellitus - the Non Obese Diabetic (NOD/ShiltJ).

In vivo IP to FITC Dextran 4kDa (FD4) measured in blood during a 4h kinetic after gavage was higher in diabetic NOD mice compared to non-diabetic NOD mice. Surprisingly, *ex vivo* IP to FD4 in Ussing chambers were not different between groups neither in jejunum nor in colon. Diabetes-induced modifications of renal excretion, transit time, feces humidity and histology failed to explain this difference. However, diabetic mice had significantly longer intestine compared to non-diabetic mice and intestinal length positively correlated with *in vivo* IP.

Our results demonstrate that depending on technic used to address IP, results can be different even if using the same marker. This difference could be explained by confounding factors affecting *in vivo* measurements whereas *ex vivo* IP is addressed in a controlled surface and thus correspond to the ability of luminal antigen to cross the intestinal epithelium. FD4 concentrations measured in blood after gavage reflect systemic exposure to luminal content rather than IP *stricto sensu*.

INTRODUCTION

There are multiple methods for the determination of intestinal permeability (IP) including *in vivo* and *ex vivo* measurements. First, *in vivo* methods are often used due to the simplicity of protocol and the absence for the need of complex device. Indeed, several non-metabolized molecules can be used to determine their passage across intestinal barrier *in vivo* in animal and human. The individual is receiving the molecule *via* the oral route and after a fixed time, blood or urine is collected and the marker is detected according to its properties (radioactivity, fluorescence, etc). Thanks to distinct chemical characteristics and degradation process by intestinal microbiota, different markers can be used to assess permeability of different intestinal segments ¹. Another possibility to assess intestinal permeability is by dosing serological markers without any previous gavage, as for example zonulin-1 ² or endotoxin ³ that will represent respectively intestinal homolog of a *Vibrio cholerae* enterotoxin that reversibly increase IP and a marker of intestinal lipopolysaccharide translocation in the blood. However, the relevance of using those markers as indicators of IP is still under debate. Last, *ex vivo* experiments can be used to determine IP. Tight junctions (TJ) proteins, sealing the paracellular pathway, can be observed through microscopy or in protein extracts of tissues. Tremendous amount of tight junctions proteins have been described and various mechanisms of compensation identified ⁴. Analysis of TJ proteins represents an interesting complement to identify the mechanisms involved when intestinal flux are modified but their analyze alone can not lead to any conclusion regarding the physiological process of IP. Ussing chambers are a well-established *ex vivo* method to analyze permeability of different tissues ⁵. However, they are more invasive, since biopsies are necessary. Looking at the functionality of a tissue, as in Ussing chambers, is of great advantage since it is performed in a controlled surface avoiding the confounding factors involved in *in vivo* experiment per gavage i.e. intestinal transit, renal excretion, intestinal length.

Increased intestinal permeability has been highlighted as a cause or a consequence in intestinal but also extra-intestinal disorders. A condition in which increased intestinal permeability is documented is type 1 diabetes (T1D) - an autoimmune disorder characterized by hyperglycemia involving pancreatic islet cells antibodies and destruction of β -cells producing insulin. Intestinal hyperpermeability is observed in human patients but also in rodents models (NOD mice, BioBreeding Diabetes Prone rats) even before the onset of the disease ⁶⁻⁸. These facts nourished the debate if intestinal hyperpermeability is triggering the onset of T1D by increasing the translocation of luminal antigen that might contribute to

destruction process of insulin producing cells. However, until today no consensus is found. To our knowledge, all studies investigating IP in T1D (human and animal models) have been performed *in vivo*. In our laboratory, we investigate IP principally through two different methods: *in vivo* by FITC-Dextran 4 kDa measurement in plasma 4h after oral gavage and *ex vivo* in Ussing chambers. We aimed to further characterize the defect of intestinal permeability in a mouse model of T1D - the Non Obese Diabetic Strain (NOD) - using both *in vivo* and *ex vivo* methods.

RESULTS

Results of intestinal permeability measurements in vivo and ex vivo by FD4 are conflicting

Consistently with literature, passage of FD4 through the intestinal barrier into the plasma had a tendency to be increased in NOD mice with overt diabetes compared to non-diabetic (ND) NOD mice four hours after FD4 gavage ($1.370 \mu\text{g/mL} \pm 0.17$ vs 0.919 ± 0.12 , $p=0.0809$, Figure 1A). However, intestinal permeability assessed in Ussing chambers did not show any increased permeability for FD4, neither in small intestine (SI) (jejunum) nor in colon (Figure 1B). We wondered if differences are due to modification in the kinetic of passage from intestinal lumen to blood. Kinetic of fluorescence intensity (FI) during *in vivo* intestinal permeability assessment were monitored by repeated blood collection during 4h *post* FD4 gavage. FI in blood of diabetic NOD mice was increased all along the experiment and a significant difference is observed 1h after gavage ($11.975 \mu\text{g/mL} \pm 3.61$ vs 3.340 ± 0.81 , $p<0.01$, Figure 1C). The area under the curve (AUC) of FI during 4h was increased for diabetic NOD mice in comparison to non-diabetic mice (1882 ± 556.8 vs 670.4 ± 167.3 , $p=0.0499$, Figure 1D).

Urinary FD4 excretion is higher in diabetic NOD mice

In order to decipher the origin of the difference of *in vivo* and *ex vivo* measurements, we questioned a potential defect of FD4 excretion in urine of diabetic compared to non-diabetic (ND) mice. To do so, intestinal pathway was bypass using intravenous injection of FD4. Diabetic NOD mice had elevated excreted quantity of urine after 1 and 2 hours (Figure 2A) with similar FD4 concentrations in urine the first hour and lower FD4 concentration the second hour (Figure 2B). As a consequence, quantity of FD4 excreted in urine was higher in diabetics the first hour and similar the second one (Figure 2C). The consequence of this higher urinary excretion of FD4 by diabetic mice is lower FD4 concentration in plasma of diabetic mice 2h after intravenous injection (Figure 2D).

Additionally we addressed fecal humidity and transit time, which both were similar between diabetic and non-diabetic NOD mice (Figure 3).

Intestinal length is increased in diabetic NOD mice

We wondered if the intestinal morphology was modified by diabetes in NOD mice compared to non-diabetic (ND) mice. Surprisingly at macroscopical level, small intestine ($45.24 \text{ cm} \pm 0.72$ vs 41.53 ± 0.76 , $p=0.0009$, Figure 4A) and colon ($9.80 \text{ cm} [8.50; 10.60]$ vs $8.75 [8.5; 9.43]$, $p=0.0306$, Figure 4B) of diabetic NOD mice were significantly longer than intestine of non-

diabetic NOD mice, but we did not observe any further differences at microscopic level. We did not observe any particularity or abnormality by microscopic screening of duodenal, jejunal, ileal or colonic tissue. Villi length, crypt depths and intestinal circumference were measured and no differences were observed between diabetic and non-diabetic NOD mice (Figure 4C-F)

Small intestine length is positively correlated with AUC of FD4 concentrations in blood

Total intestinal length is correlated with the area under the curve of FD4 concentration during 4h after gavage (Pearson correlation, $r=0.6659$, $R^2=0.4434$, $p=0.0253$, Figure 5).

Ex vivo paracellular intestinal permeability to FSS is decreased in diabetic mice

In order to further characterize intestinal permeability, we used Fluorescein Sodium Salt (FSS) and Horse Radish Peroxidase (HRP) as specific para- and transcellular marker respectively in Ussing chambers. Paracellular permeability to FSS was significantly decreased in small intestine (jejunum) of diabetic mice compared to non-diabetic (ND) mice ($44.859 \text{ ng/cm}^2/2\text{h} \pm 11.05$ vs 217.96 ± 76.50 , $p<0.05$, Figure 6A). Short circuit current (I_{sc}) in colon was significantly increased in diabetic NOD mice compared to non-diabetic NOD mice ($-13.335 \text{ } \mu\text{Acm}^2 \pm 1.59$ vs -25.266 ± 6.39 , $p<0.05$, Figure 6B). Transcellular permeability to HRP was not modified; neither in jejunal nor in colonic tissue (Figure 6C). Intact HRP as a measure of non-lysosomal transcellular pathway was not affected either (Figure 6D).

Diabetic state is not associated with inflammatory profile

We wondered if diabetes had an effect on inflammatory state in the intestine. We screened several cytokines (TNF α , Lipocalin-2, IL-10, IL-17 and IL-22) in lysate of colon and jejunum (small intestine: SI). As expected cytokine levels in colonic tissues were higher. IL-10, IL-17 and IL-22 were non-detectable in lysate of SI tissue. There were no diabetes-induced modifications in cytokine concentrations (Figure 7).

DISCUSSION

We here demonstrate that using the same marker FITC-Dextran 4 kDa (FD4) *in vivo* and *ex vivo* to measure IP can give different results in a type 1 diabetes model: the non-obese diabetic mice.

In our study, we compared NOD diabetic *vs* non-diabetic mice. This is an advantage in comparison to other studies in which different mouse/rat strains were compared, for example NOD *vs* non-obese resistant (NOR) mice or BioBreeding diabetes-prone (BBDP) *vs* BioBreeding diabetes-resistant rats (BBDR) ^{8,9}.

We used FD4 to assess intestinal permeability *in vivo*. As most laboratories take plasma at 4h after gavage, when FD4 had time to transit all along the intestinal tract ¹⁰, we choose to follow this method. We observed a tendency for an increase in FD4 concentration in plasma at 4h after gavage in diabetic mice that is consistent with literature. Indeed, intestinal permeability has been described to be increased by auto-immune diabetes in rodents and in human ^{6-8,11}. During *ex vivo* jejunal (representative of small intestine) and colonic permeability measurements in Ussing chambers we used the same marker i.e. FD4. Surprisingly, *ex vivo* FD4 flux was not different between diabetic and non-diabetic NOD mice. This result suggests that the increased FD4 concentration in blood observed *in vivo* is not the result of increased FD4 flux in jejunum and/or colon and as such excludes an increase of IP *stricto sensu* in auto-immune diabetes. Indeed, the proper definition of IP is “the facility with which intestinal epithelium allows molecules to pass through by non-mediated passive diffusion” expressed in cm/sec ¹ and as such need to take into account the exposed surface which cannot be done in *in vivo* experiments. To decipher differences in kinetic of FD4 absorption in the mouse model, blood samples were taken at different time points during the 4h-period after FD4 gavage and FD4 concentrations measured. Although FD4-flux in Ussing chambers were similar in tissue of diabetic and non-diabetic mice, all along the *in vivo* experiment FD4 concentrations in blood were higher in diabetic compared to non-diabetic mice and significantly different at 1h after FD4 gavage. As a result, AUC of FD4 is higher in diabetic mice compared to non-diabetic mice. Those results highlight the interest to follow the kinetic of FD4 in blood rather than measuring FD4 concentration in plasma at a unique time point. Of note, Woting and Blaut observed that absorption kinetics is highly depend on mouse strain ¹².

We then wondered if urinary excretion of FD4 was modified. It is largely known that diabetes comes along with renal complications. Hyperglycemia leads to polyuria and 80% of

persons diagnosed with diabetes mellitus have lower urinary tract complications, as for example nephropathy that at the ultimate stage can lead to a defect of urinary excretion process and request dialysis intervention ¹³. However, our results showed that the quantity of FD4 excreted in urine of diabetic mice is higher the first hour and the same the second hour. This effect is mainly driven by increased volume of urine excreted by diabetic mice compared to non-diabetic rather than higher concentration of FD4 excreted. In those experiments of urinary excretion, bypassing intestinal pathway, higher quantity of FD4 excreted in diabetic mice is associated with lower FD4 in plasma 2H after gavage. Those results excluded a defect of renal excretion of FD4 in diabetic mice to explain higher FD4 concentration in plasma after FD4 oral gavage (*in vivo* permeability assessment).

Transit time and feces humidity were not different between diabetic and non-diabetic mice. However, in literature enhanced intestinal transit, measured by charcoal progression after 20 min in the intestinal tract, is described in NOD mice with long-term diabetes (4-5 weeks) ¹⁴. In insulin-dependent diabetes mellitus patients both delayed and rapid intestinal transit has been documented ¹⁵. Nevertheless, since transit time was not modified in our study transit could not explain the increased FD4 concentration in blood.

We looked in detail at intestinal morphology by histology. Surprisingly no aberrant structures indicating inflammation or epithelium modifications have been observed. Villi length and crypt depth were similar between diabetic and non-diabetic mice. Neu et al. observed increased mucosal crypt depth in BioBreeding diabetes-prone vs BioBreeding diabetes-resistant rats ⁹. However, as already pointed out at the beginning of the section discussion, they compared two different strains (BBDP vs BBDR) whereas in our study we compare same strain but different diabetic state. In streptozotocin-induced diabetic rats increased wall stiffness ¹⁶ and increased thickness of intestinal mucosa ¹⁷ have been observed. Circumference of ileum was increased and contraction force of intestinal smooth muscles were decreased in diabetic rats ¹⁸. Another study reported that colonic smooth muscle from RIP-I/hIFN β diabetic mice had similar contractile responses with control mice ¹⁹. In human, 75% of diabetes mellitus patients (T1D and T2D) suffer from gastro-intestinal symptoms ²⁰. Abnormal motility has been described in patients suffering from auto-immune diabetes with conflicting results, some report intestinal hypermotility ²¹ others hypomotility ²². In our study, we do not observe any difference in circumference of small (duodenum, jejunum, ileum) and large intestine between diabetic and non-diabetic mice excluding a major role of tonus to explain our different results in *in vivo* and *ex vivo* measurements for FD4.

The most striking result of our study was the elongation of intestine in diabetic NOD mice. Interestingly, increased intestinal proliferation, intestinal weight and length have previously been described in streptozotocin- and alloxan-induced diabetes^{23,24}. The increase of intestinal length means an increase of absorptive surface that seems to be a main contributor to the observed higher FD4 concentration in plasma of diabetic mice compared to non-diabetic mice.

We aimed to further characterize intestinal permeability in our model in Ussing chambers using FSS and HRP as respective markers of para- and trans-cellular permeability²⁵. Small inert molecules with a molecular weight below 600 Da can pass the epithelial barrier between cells *via* the tight junctions²⁶. Horse Radish Peroxidase (HRP) is a bigger molecule (44 kDa) which can pass the intestinal epithelial barrier by transcellular route via endocytosis. For that, it serves as a marker to measure transcellular permeability. Surprisingly, in jejunum, paracellular permeability to FSS was significantly decreased in diabetic mice whereas transcellular permeability to HRP was not modified (intact and total HRP). FD4 can be considered as a marker of transcellular permeability due to its molecular weight (4 kDa) and as it acts like HRP and not FSS.

Short current circuit (Isc) was significantly increased in colon of diabetic mice and might reflect an increased Cl⁻ secretion into the lumen triggering H₂O flux in the lumen²⁷. However, we did not observe differences in fecal humidity and intestinal transit time excluding a major effect on water fluxes.

In our model, diabetic state was not associated with intestinal inflammation. So we could exclude any direct effect of inflammation on modified intestinal morphology and permeability, since inflammation comes along with increased intestinal permeability²⁸

During *in vivo* measurements FD4 concentrations are increased in diabetic mice as early as 15 min after gavage that could involve gastric and duodenal permeability since FD4 is increased shortly after gavage and the FD4 bolus is still in the upper intestinal part, it would be interesting to assess additionally gastric and duodenal permeability. Indeed, in insulin-dependent diabetes patients abnormal duodenal chyme transport has been observed²⁹. Even though, the accurate characterization of IP in different fragments of small intestine is of interest, this is not the object of our study. Here our objective was to emphasize that *ex vivo* and *in vivo* measurement of FD4 transport by intestine can lead to conflicting results and we used jejunum as a representative fragment of small intestine.

In summary, our work highlights that the assessment of intestinal permeability is complex and a lot of confounding factors have to be considered when setting up the experiment. On one side, *in vivo* experiments do not allow to control the absorptive surface, neither intestinal circumference nor intestinal length, are known in the experimental conditions. Furthermore, one has also to consider that *in vivo* intestinal permeability measurements are highly dynamic – depending on transit, excretion and could be fluctuating in time. One point plasma measurements are difficult to consider as representative. Overall, *in vivo* measurements are reflecting more likely the exposition of the organism to the luminal marker in circulation at one time point, rather than the intestinal permeability as such in its *stricto sensu* definition. On the other side, Ussing chamber allow to control surface. This allows the researcher to analyse different intestinal segments and to use different molecular markers. Ussing chambers allow to measure real permeability, which is “the facility with which intestinal epithelium allows molecules to pass through by non-mediated passive diffusion” expressed in $\text{cm}/\text{sec}^{-1}$. However, tissues are outside of the organism and effects of innervation and blood circulation are depleted and additional observations and experiments are necessary to describe the physiologic importance of observed modifications *ex vivo*.

MATERIALS AND METHODS

Mouse model

All experimental protocols were approved by local Animal Care Use Committee (Comité d'Ethique de Pharmacologie-Toxicologie de Toulouse - Midi-Pyrénées, France) registered as N°86 at the Ministry of Research and Higher Education (N° 0029/SMVT), and conducted in accordance with the European directive 2010/63/UE. NOD Shilt/J strain (Charles River, France) were housed by five per cage. Female NOD mice were chosen for the experiment since they develop more rapidly diabetes than male NOD mice (Jackson Laboratory Physiologic Data Sheet). Mice were kept at a constant temperature ($22 \pm 1^\circ\text{C}$) and maintained on a 12:12 h light/dark cycle (lights on 8h00 am) on Specific and Opportunistic Pathogen Free (SOPF) conditions. Normal diet (Harlan2018, Envigo, Gannat, France) and water were available *ad libitum*. Blood glucose levels of NOD Shilt/J mice were monitored weekly from the tip of the tail vein with a glucose meter (AccuCheck, Roche, Mannheim, Germany). Mice with non-fasted glycaemia superior to 250 mg/dL were considered as diabetic according to Jackson Laboratory Physiologic Data Sheet. After one to four weeks posterior to diabetes diagnosis, diabetic mice and non-diabetic mice from same cage were attributed either to *in vivo* or *ex vivo* intestinal permeability assays. Three independent batches of animals were analyzed.

Intestinal permeability in vivo – FITC-Dextran 4 kDa (FD4)

Mice were fasted for 3h prior to intragastric gavage with FITC-Dextran 4kDa (750 $\mu\text{g/g}$ bodyweight in tap water). Small blood samples were obtained at the tip of the tail vein via microheamatocrit capillary tubes (Hirschmann, Eberstadt, Germany) before and 15 min, 30 min, 60 min, 2h, 3h, 4h after gavage. At the end of the procedure mice were euthanized by dislocation and blood was harvested intracardiac for plasma. Blood samples were diluted 1/20 and plasma 1/10 in NaCl 0,9%, and Fluorescent Intensity (FI) measured by excitation: 485 nm/emission: 525 nm using an automatic Infinite M200 microplate reader. FI in blood was corrected by FI measured before gavage.

Intestinal permeability ex vivo - Ussing chambers

Intestinal permeability was assessed as previously described ²⁵. Briefly, jejunal (as representative for small intestine: SI) and colonic fragments were mounted in Ussing chambers (Physiologic Instruments, San Diego, CA, USA). Tissues were bathed 2h with oxygenated thermostated Krebs solution (Sigma, Saint Quentin Fallavier, France). Horse Radish Peroxidase 400 $\mu\text{g/ml}$ (HRP 40 kDa; Sigma) and Fluorescein Sodium Salt 40 $\mu\text{g/ml}$ (FSS 376Da; Sigma)

or FITC-Dextran 400 $\mu\text{g/mL}$ (FD4 4 kDa, TdB, Sweden) were added to mucosal compartment. FSS and HRP were used as respective markers for para- and trans-cellular intestinal permeability.

Intestinal permeability to total HRP was determined by ELISA. Briefly, 96-wells black plates (Greiner, Les Ulis, France) were coated with 10 $\mu\text{g/ml}$ mouse polyclonal to HRP (Abcam, Paris, France), blocked with PBS-1% bovine serum albumin (BSA), incubated with serosal compartments of Ussing chamber, detected with 10 $\mu\text{g/ml}$ Rabbit polyclonal anti HRP biotin (Abcam) and revealed with SPRD-conjugated streptavidin (BD, Paris, France). Fluorescence intensity was measured 485nm/525nm using an automatic Infinite M200 microplate reader (Tecan, Männedorf, Switzerland). Intestinal permeability to intact HRP was determined in 96-wells black plates, adding TMB substrate to serosal compartments of Ussing chambers, and measuring the slope within 90 seconds at 37°C at 450 nm in automatic Infinite M200 microplate reader. Intestinal permeability to FSS and FD4 were determined by measuring FI 485 nm/525 nm using an automatic Infinite M200 microplate reader. Results of Ussing chambers measurements are presented as flux, expressed as ng/2h/cm^2 .

FD4 excretion in urine

For analysis of FD4 excretion in urine, mice were anesthetized by a mixture of ketamine 100 mg/kg of body weight and xylazine 10 mg/kg of body weight and maintained anesthetized by jugular catheter perfusion with 20mg/kg/h ketamine and a flow of 0.1ml/h. 10 mg FD4 was injected in jugular vein (100 mg/mL), arterial pressure was monitored in femoral artery. Urine was collected through a catheter inserted in bladder at 1h and 2h after FD4 injection. FD4 concentration was measured by FI. After 2h mice were euthanized by cervical dislocation, blood was taken for measurements of FI in plasma.

Histological analysis of intestinal morphology

Intestinal fragments of duodenum, jejunum, ileum and colon were fixed in 4% formalin and included in paraffin. Paraffin sections were stained with Hematoxylin and Eosin (Eosine aqueuse 1% Labo modern, France). Tissue were screened for morphological abnormalities. Intestinal diameter, crypt depth and villi length were measured (Nis Element-Ar, Nikon System-Elements-Advanced research, microscope Nikon Eclipse 90 i, France).

Fecal humidity and Transit time

Feces of diabetic and non-diabetic mice were collected and regrouped by condition, weighed, dried at 37°C for one week and weighed again. Lost weight was considered as water

content. In the morning, mice were isolated in clean cages without bedding material but tissue for 45 min without food. Afterwards feces were numbered.

Cytokine measurements

Cytokines were measured in jejunal (representative of small intestine: SI) or colonic fragments suspended in RIPA buffer (0.5% deoxycholate, 0.1% SDS and 1% Igepal in TBS) containing complete anti protease cocktail (Sigma). Jejunal and colonic protein concentrations were measured using BCA uptima kit (Interchim, Montlucon, France). TNF α , Lipocalin-2, IL-10, IL-17 and IL-22 in lysate of jejunal or colonic fragments were assayed using commercial ELISA kits (R&D Systems, Lille, France) and expressed as pg/mg of protein.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.04 (GraphPad Software, La Jolla, CA, USA). Results for single comparisons were displayed as box plots [min to max] and analyzed using Student's unpaired t-test (two-tailed) or Mann-Whitney test (two-tailed) after prior Shapiro-Wilk Normality test and F-Test to compare variances. Multiple measurements in time or in different tissues were displayed either as kinetics with SEM or box plots [min to max] compared per family by Holm-Sidak posttest after a significant two-way ANOVA. Differences were considered significant for $P < 0.05$. Results in text were described as NOD diabetic mean \pm SEM vs. NOD non-diabetic mean \pm SEM for normally distributed samples and as median, [25%-quartile; 75%-quartile] in other case.

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ACKNOWLEDGEMENTS

CD and MB were partially supported by a grant from the "Fondation pour la Recherche Médicale" (grant number DEQ20170336759).

AUTHORS CONTRIBUTION

SM, CD, HID, MB and VT designed the work. HID, SM, VB and MB carried out the experiments. HID and SM wrote the manuscript. HID, MB, VB, VT, CD and SM approved the submitted manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

LEGENDS

Figure 1 *In vivo* and *ex vivo* measurements of intestinal permeability to FITC-Dextran 4 kDa (FD4) give different results. **(A)** Plasmatic FD4 concentration 4h after gavage by 750 μg FD4/g bodyweight, unpaired students t-test, $p=0.0809$, $n=4-5$. **(B)** Jejunal (small intestine: SI) and colonic permeability to FD4 (400 $\mu\text{g}/\text{mL}$ in serosal compartment) measured in Ussing chambers, $n=4-5$. **(C)** Kinetic of FD4 fluorescence in blood during 4h after gavage by 750 μg FD4/g bodyweight, repeated-measurement two-way ANOVA, $** p<0.01$, $n=5-6$. **(D)** Area under the curve (AUC) of FD4 in blood during 4h after FD4 gavage, unpaired students t-test, $p=0.0499$, $n=5-6$.

Figure 2 Urinary excretion of FITC-Dextran 4 kDa (FD4) is higher in diabetic NOD mice. FD4 (10 mg/mice) was injected in jugular vein in anesthetized mice **(A)** urinary volume (μL) measured 1h and 2h after intravenous injection of FD4 harvested in bladder during the experiment in anesthetized mice, $n=2-3$. **(B)** FD4 concentrations in urine ($\mu\text{g}/\text{mL}$), $n=2-3$. **(C)** Quantity of FD4 excreted as calculated from FD4 concentrations measured in urine multiplied by urine volume, $n=2-3$. **(D)** FD4 concentrations measured in plasma harvested 2h after FD4 intravenous injection, $n=2$.

Figure 3 Intestinal transit and humidity in feces are not affected by diabetes in NOD diabetic mice. **(A)** Intestinal transit, measured as number of feces/mice during 45 minutes, $n=7-14$. **(B)** Fecal humidity calculated as the ratio of fresh feces weight (one cage) and one week at 37°C dried feces weight, $n=2$.

Figure 4 Macroscopical and microscopical and characterization of small intestine and colon in diabetic vs non-diabetic NOD mice. **(A)** Small intestine length (cm), unpaired students t-test, $p=0.0009$, $n=25-26$. **(B)** Colon length (cm), Mann-Whitney test, $p=0.0306$, $n=25-26$. **(C-D)** representative cross sections of 4%-formalin, paraffin-embedded haematoxylin/eosin stained jejunal, objective 2x **(E-F)** and colonic tissue, objective 10x **(C-D)** tissue $n=5$.

Figure 5 Total intestinal length (cm) is positively correlated with the area under the curve (AUC) of FD4 concentrations during the 4h after FD4 gavage, pearson correlation, $r=0.6659$, $R^2=0.4434$, $p=0.0253$, $n=11$.

Figure 6 Measurements of intestinal permeability in Ussing chambers in jejunum (small intestine: SI) and colonic tissue. **(A)** Paracellular flux ($\text{ng}/\text{cm}^2/2\text{h}$) of Fluorescein Sodium Salt (FSS), 330 Da, $40 \mu\text{g}/\text{mL}$ in serosal compartment, two-way Anova, Sidaks multiple comparison post-test, $*p<0.05$, $n=8-12$. **(B)** Short circuit current (I_{sc} , $\mu\text{A}\cdot\text{cm}^2$), two-way Anova, Sidaks multiple comparison post-test, $*p<0.05$, $n=7-10$. **(C)** transcellular flux ($\text{ng}/\text{cm}^2/2\text{h}$) of total Horse Radish Peroxidase (HRP), 44 kDa, $400 \mu\text{g}/\text{mL}$ in serosal compartment, $n=8-13$. **(D)** transcellular flux ($\text{ng}/\text{cm}^2/2\text{h}$) of intact Horse Radish Peroxidase (HRP), 44 kDa, $400 \mu\text{g}/\text{mL}$ in serosal compartment, $n=6-8$.

Figure 7 Cytokine concentrations in colonic and jejunum (small intestine: SI) lysate were similar between diabetic and non-diabetic NOD mice. Cytokine concentrations in (pg/mg protein) **(A)** $\text{TNF}\alpha$, $n=5$ and **(B)** Lipocalin-2, $n=5$ in jejunum (small intestine: SI) and colon. **(C)** IL-10, $n=5$ **(D)** IL-17, $n=5$ **(E)** IL-22, $n=5$, in colon.

Figure 1

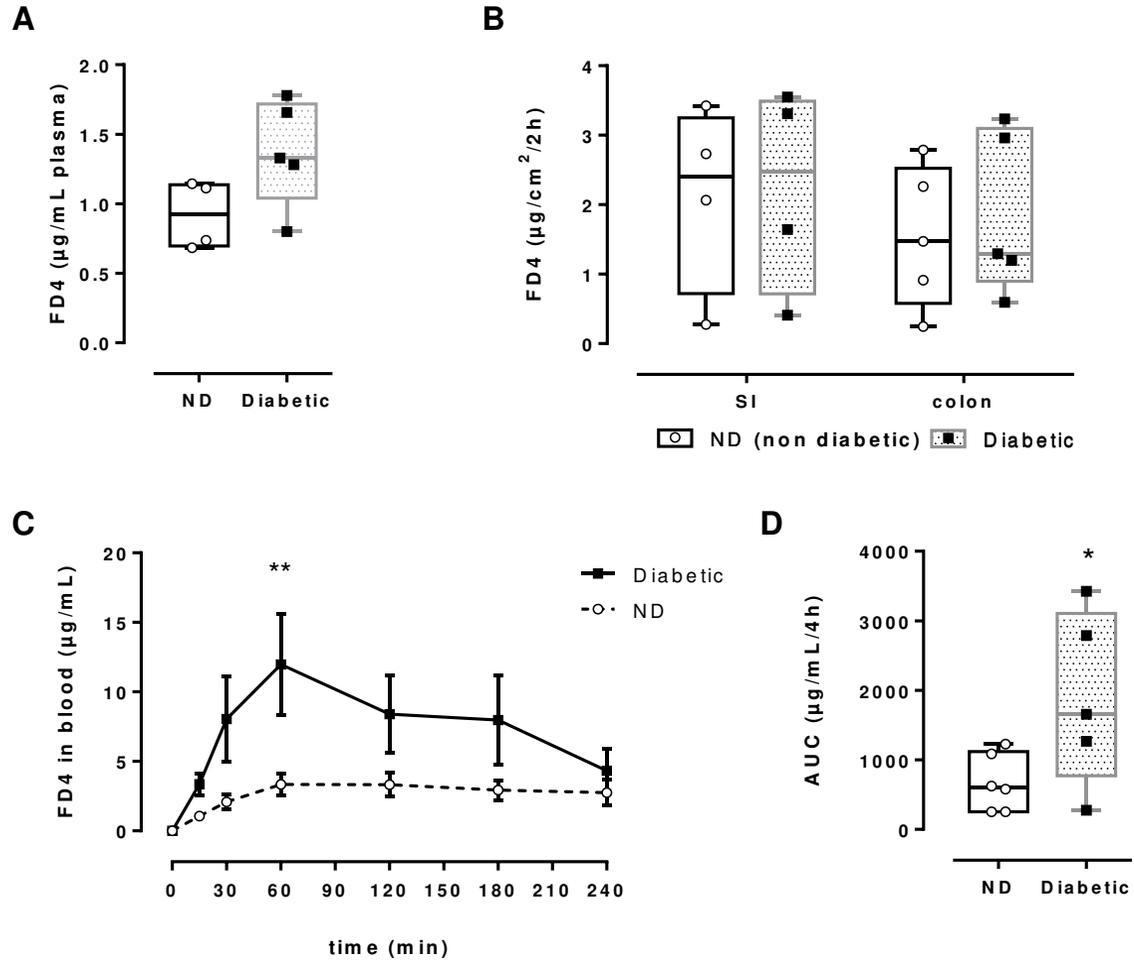


Figure 2

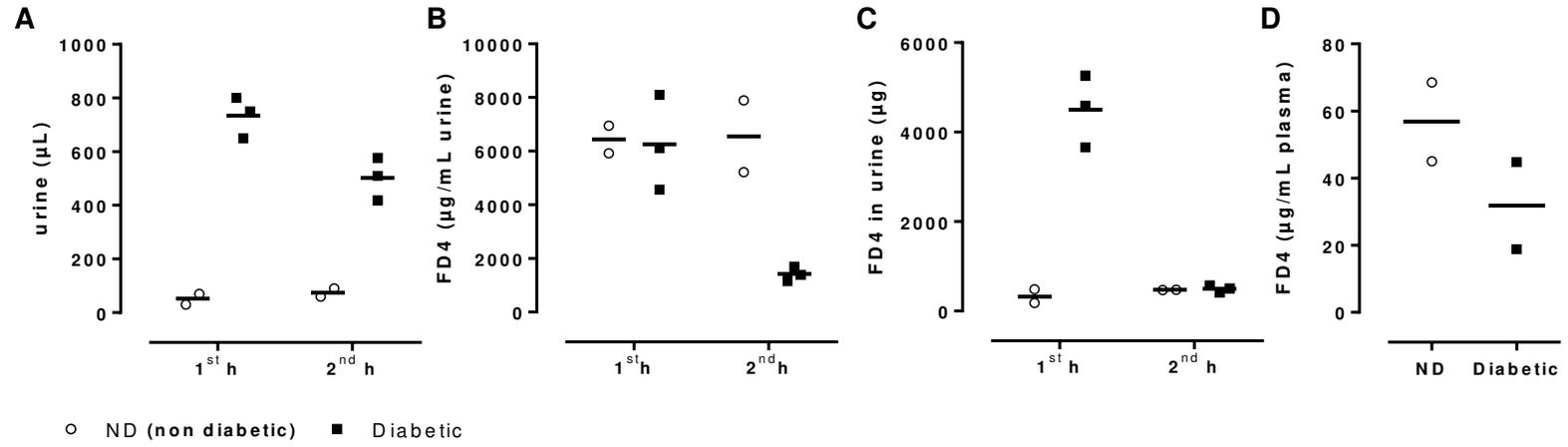


Figure 3

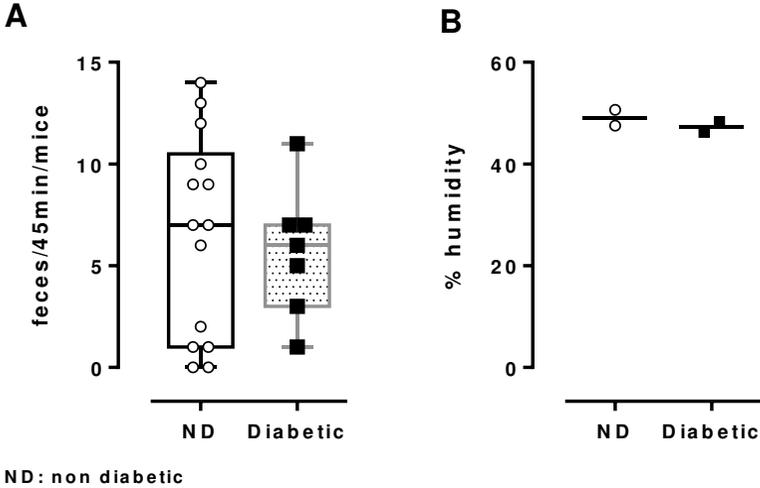


Figure 4

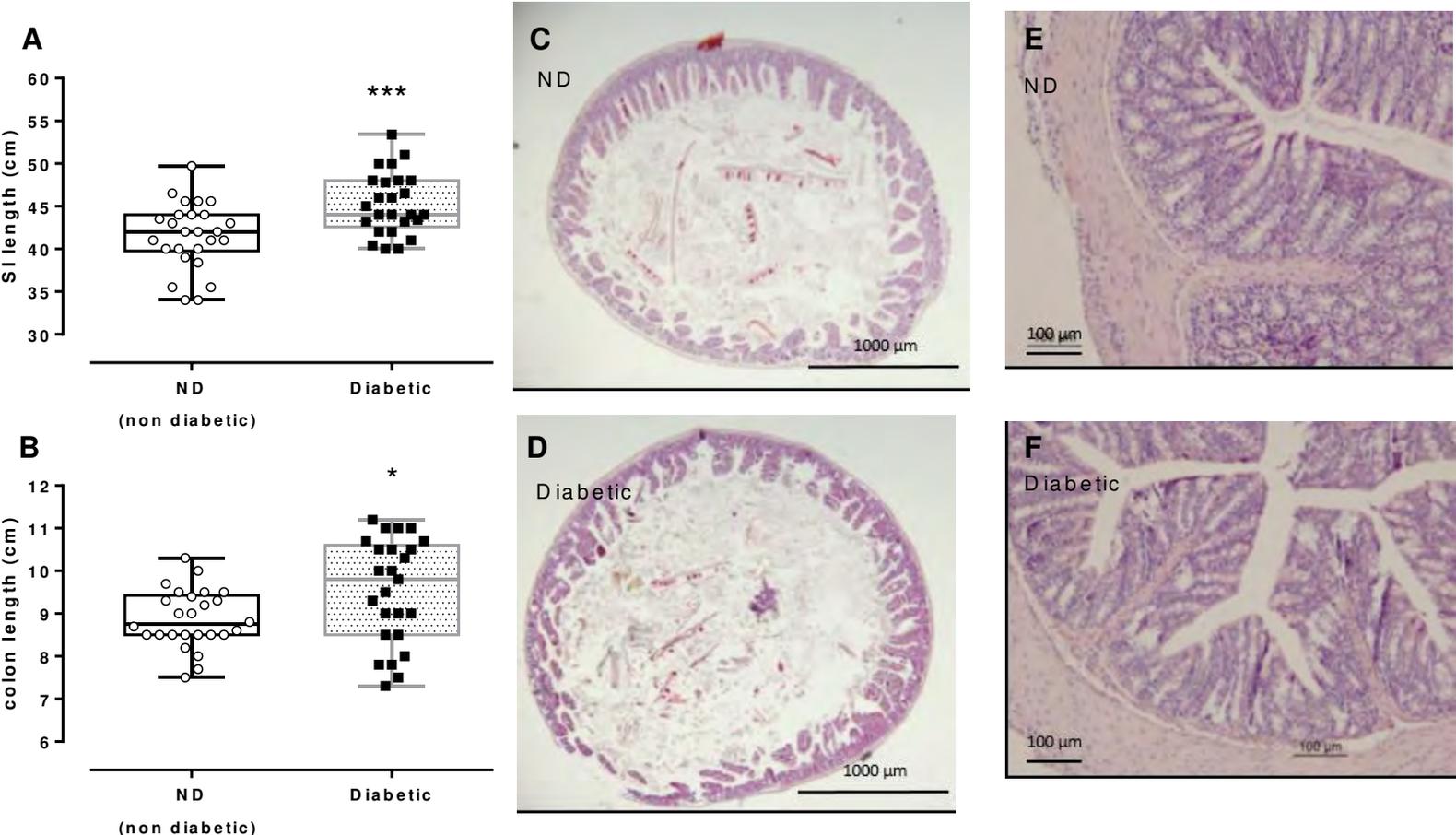


Figure 5

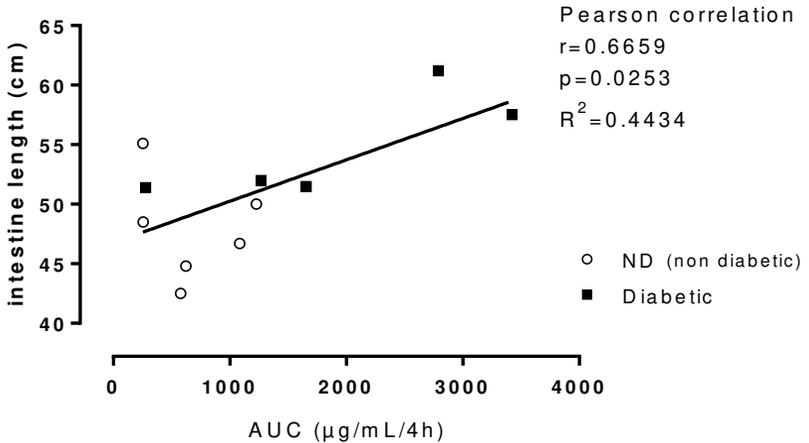


Figure 6

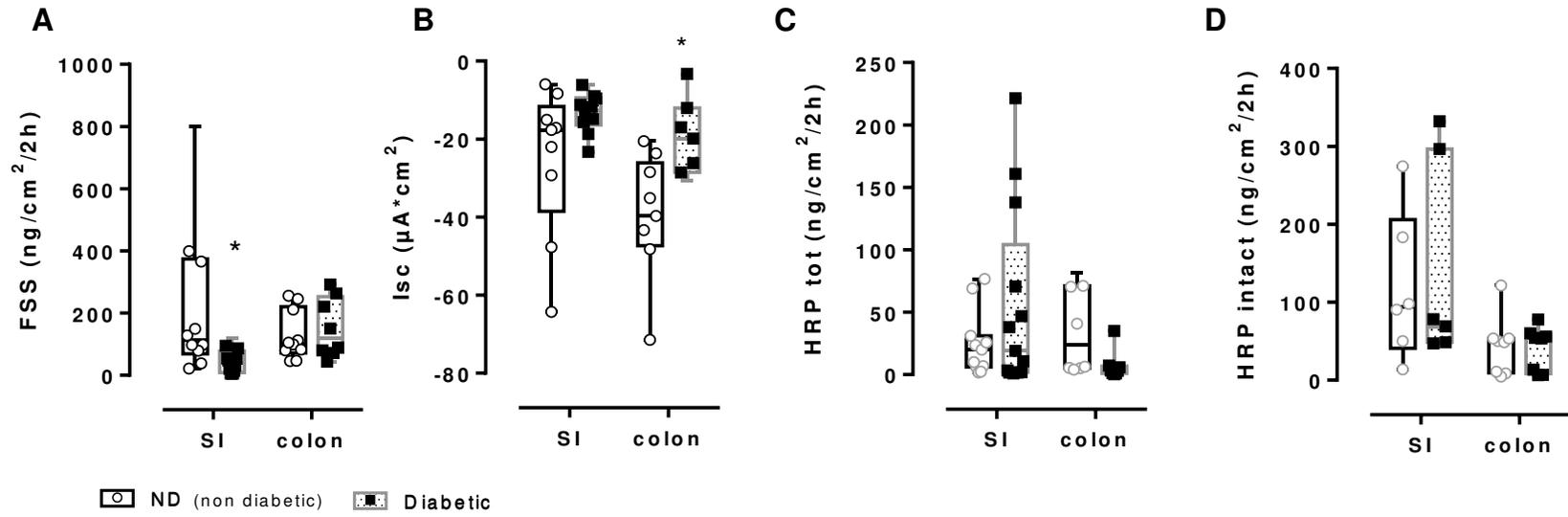
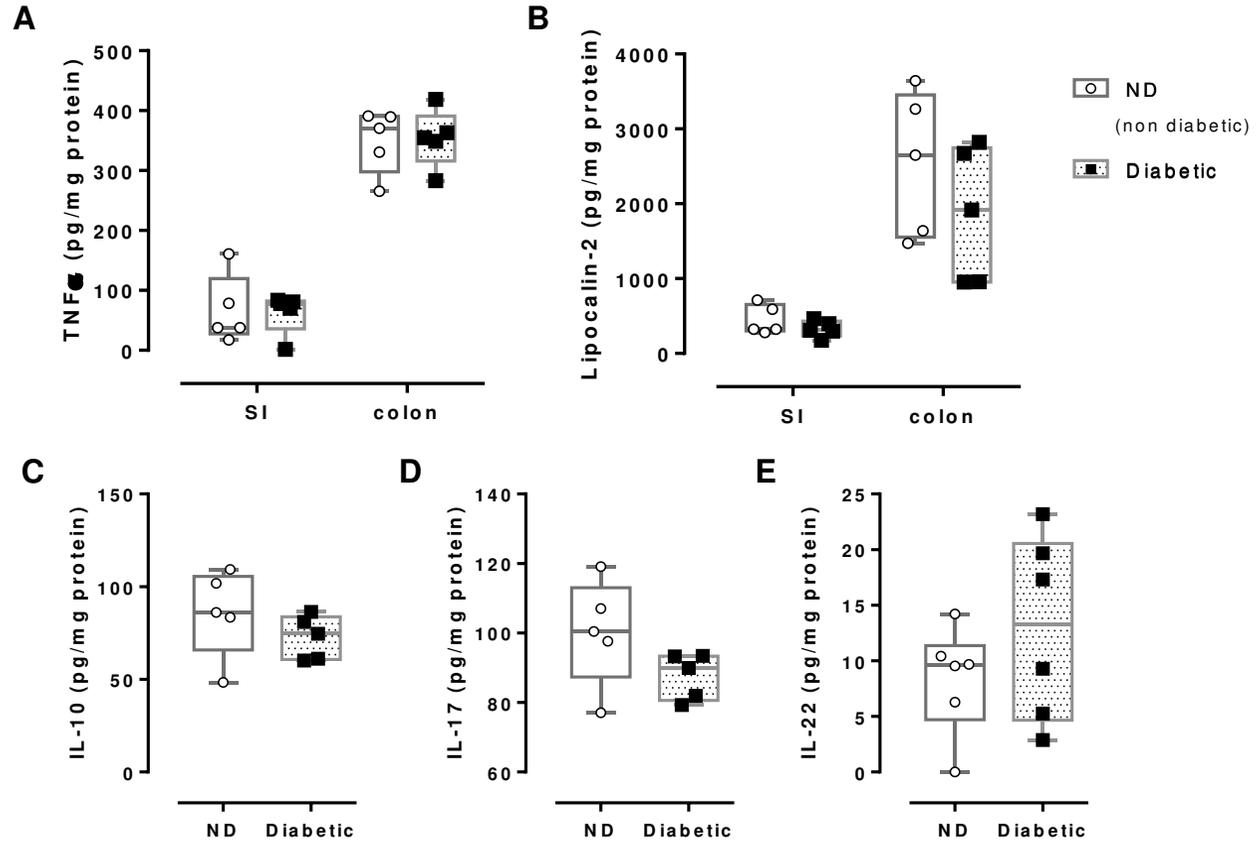


Figure 7



Additional Discussion

The original article published by Thaïss and colleagues in 2018 in the journal *Science* is entitled “Hyperglycemia drives intestinal barrier dysfunction and risk for enteric infection”. The question if hyperglycemia is triggering intestinal barrier dysfunction (including intestinal hyperpermeability) is of great interest in our model. The authors are principally working with a model of type 1 diabetes induced by streptozotocin (STZ). One has to note that STZ is entering the cell via GLUT2 (Lenzen, 2008; Wang and Gleichmann, 1998), which is not only expressed in β cells of pancreas but also on intestinal epithelial cells. Thus, a direct effect of STZ on the observed intestinal barrier dysfunction cannot be excluded. Additionally, STZ is also used in gut cancer (Clamon et al., 1987; Welt et al., 2003), emphasizing its action on intestinal epithelial cells and thus an impact on intestine. STZ is also used as chemotherapeutic agent. A clastogenic effect of STZ on human colon cancer cell lines has been described (Bolzán and Bianchi, 2003). In their paper, Thaïss *et al.* describe that mice with a partial knock-out of GLUT2 on intestinal epithelial cells (GLUT2 ^{Δ IEC}) were resistant to STZ-induced transcriptomic and tight junctional changes despite persisting hyperglycemia. They conclude that hyperglycemia is reprogramming via GLUT2 the intestinal transcriptome. However, these observations are more in favor for the hypothesis that STZ is affecting directly the intestinal epithelial cells. Indeed, disturbance of glucose transporters in the intestinal epithelial cell could lead to reorganization or even loss of cell polarization and an ectopic expression of SGLT1 to compensate the knock-out. Perhaps glucose could also enter into the epithelial cell via SGLT1, a glucose transporter. (Kellett and Brot-Laroche, 2005).

The authors use many different models to support their hypothesis. Indeed, they have data on STZ-induced hyperglycemia, *db/db*, *ob/ob*, *Akita* and HFD mice. It is important to note that in these models, hyperglycemia is linked with obesity and inflammation. It is difficult to disentangle the effects of these three phenomenon. Indeed, they are highly interwoven and influenced one by another. The authors present only data on immune system of STZ treated mice, but not of *db/db*, *ob/ob*, *Akita* and HFD mice, which would be interesting for the comprehension of increased pathogen load in all five models. Our work was carried out in non-obese diabetic (NOD) mice. There is no interference with obesity. Additionally we showed that hyperglycemia was not associated with inflammation. So the NOD model would have been an interesting plus in the work of Thaïss *et al.*

Even though, we were not convinced that this paper clearly demonstrated that hyperglycemia drives intestinal hyperpermeability the role of hyperglycemia on intestinal

permeability was of particular interest in our model of NOD diabetic mice. We set up the following experiment to confirm a potential role of high glucose on intestinal permeability in Ussing chambers. Intestinal permeability was assessed in Ussing chambers on small intestine and colon from NOD non-diabetic, diabetic, C57Bl6/J and Balbc/J mice in high-glucose (6 g/L) vs normal glucose (2 g/L) conditions. In jejunum (**Figure 25**) and colon (**Figure 26**), no increase FSS, FD4 and HRP fluxes after 2h bathing could be detected and electrical parameters were not affected neither. We had just 1 point for NOD mice in every condition, but did not observe increased passage of molecules in high-glucose conditions. So, we excluded a role of hyperglycemia in the increase of intestinal permeability measured in Ussing chambers.

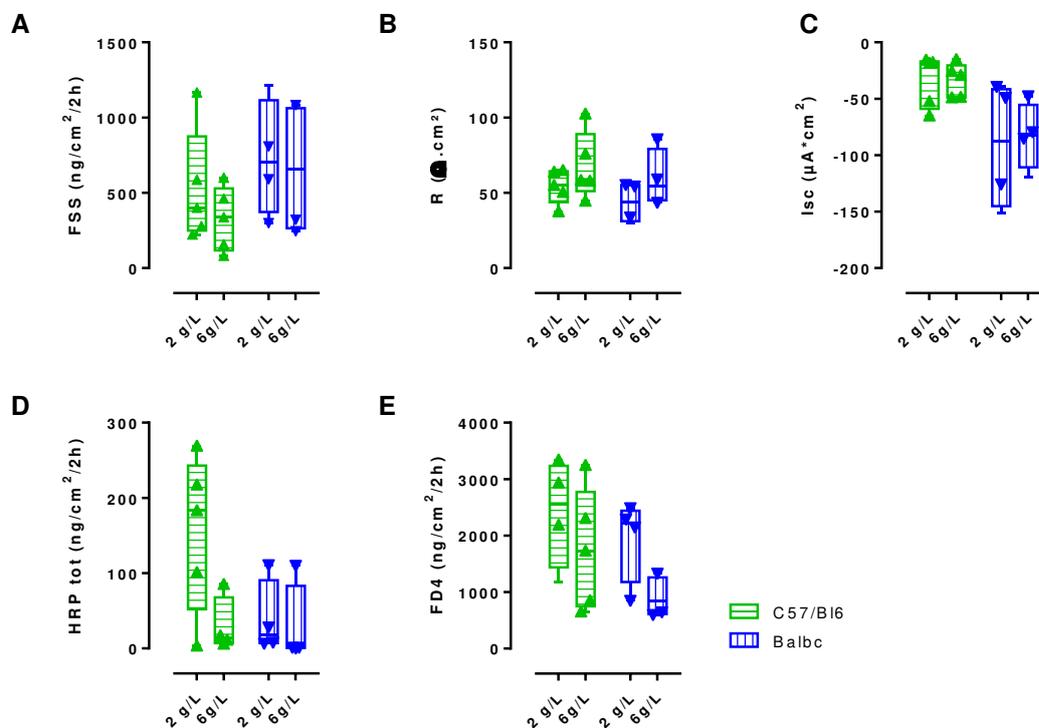


Figure 25 Jejunum permeability measurements in Ussing chambers in C57/Bl6 and Balbc mice in low (2 g/L) and high-glucose (6g/L) Krebs medium. (A) FSS-flux (ng/cm²/2h). (B) Electrical resistance (R) (Ωxcm²). (C) Short circuit current I_{sc} (μA*cm²). (D) HRP-flux (ng/cm²/2h). (E) FD4-flux (ng/cm²/2h).

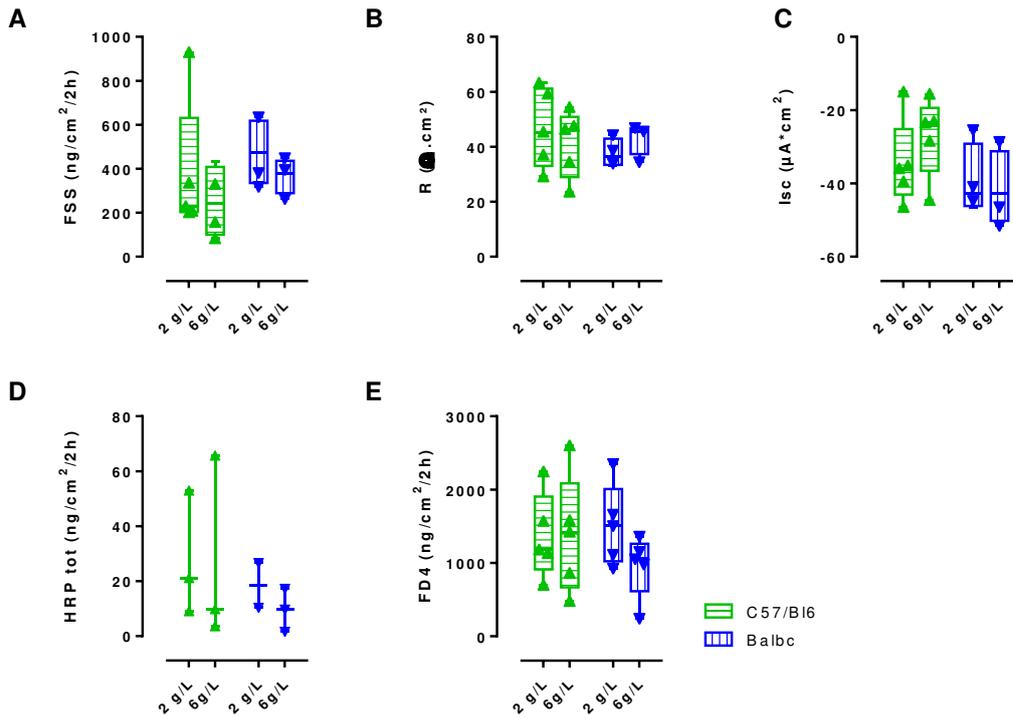


Figure 26 Colonic permeability measurements in Ussing chambers in C57/B16 and Balbc mice in low (2 g/L) and high-glucose (6g/L) Krebs medium. (A) FSS-flux (ng/cm²/2h). (B) Electrical resistance (R) (Ω·cm²). (C) Short circuit current I_{sc} (μA·cm²). (D) HRP-flux (ng/cm²/2h). (E) FD4-flux (ng/cm²/2h).

**IV. PART
GENERAL DISCUSSION**



THE INTESTINE IS PLAYING A CRUCIAL ROLE IN HEALTH AND DISEASE

Historically, MS model has been used with the objective to analyze its consequences on intestinal barrier function and as such is a reference as a rodent model of Irritable Bowel Syndrome (IBS). The interest in intestinal homeostasis gain a special attention during the last years. Indeed, intestinal homeostasis is impaired not only in intestinal disorders, but also in extra-intestinal disorders and we aimed to address this question during my PhD.

The intestine is playing a central role in our life: it is the biggest body surface in contact with the outside, transporting about 30 000 tons of food and 50 000 liter of fluids during our life, harboring the biggest immune repertoire in our organism and due to the numerous neurons often called our second brain.

The different projects of my PhD highlight the long-term consequences of stress on intestinal homeostasis and the development of diseases. Indeed, intestinal hyperpermeability is one of the first characteristics of MS-induced phenotype in young C3H/HeN male mice. Microbiota dysbiosis is observed in young MS male mice. The intestinal immune system is modified by MS in male and female mice from young age on, and remained different between MS and control throughout lifetime. Our observations on MS-induced effects in male and female young adult mice led us hence to the hypothesis that intestinal barrier dysfunction could be at the origin of later disease. Interventional studies are needed to confirm this.

In male mice, intestinal hyperpermeability induced by MS appeared from early age on. Indeed, intestinal permeability was increased from early time point on (PND15). Riba et al. reported still increased intestinal permeability at PND50 and additionally decrease in lysozyme activity (Riba et al., 2018), which were both resolved later on (PND350) (Ilchmann-Diounou et al., 2019) (**Figure 27**).

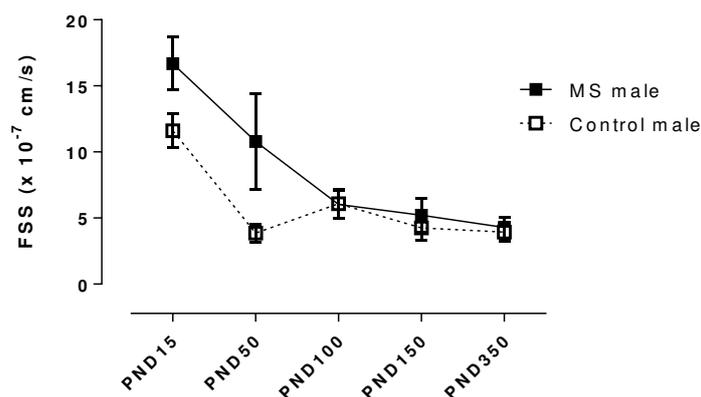


Figure 27 Jejunal paracellular permeability to FSS ($\times 10^{-7}$ cm/s) assessed in Ussing chambers in C3H/HeN male maternal separated (MS) and control mice at post-natal day (PND) 15, 50, 100, 150 and 350.

The intestinal barrier in MS male mice is also weakened by diminished lysozyme content in Paneth cells and decreased fecal lysozyme activity at PND50 and intestinal low-grade inflammation, namely increased TNF α and fecal IgA concentrations (Riba et al., 2018). The effects of MS on antimicrobial activity and on IgA concentrations were resolved in PND350 mice (*cf.* III. Part Results, First Result, Additional Data), whereas intestinal low-grade inflammation and increased IgG against commensal microbiota, which is an indicator for disturbed host-microbiota mutualism, are persisting in time (Ilchmann-Diounou et al., 2019). Metabolic disorder only appear at PND350. Additionally, microbiota dysbiosis is observed at PND50 and PND350, but evolving in time.

The establishment of life-long beneficial host-microbiota interaction takes place in the early life (Stockinger et al., 2011). After birth, the host is massively colonized and encountering microbiota for the first time. A transient disturbed barrier can have long-lasting consequences due to the potent priming mechanisms on immunity during the highly shapeable neonatal period. Increased exposition to luminal antigens (from microbiota and food origins), due to weakened intestinal barrier, in this time frame can have an impact on later disease susceptibility. Al Nabhani et al. show that a transient but robust immune reaction during weaning period have long-term effects on the resistance to immunopathology in later life, notably colitis and allergy (Al Nabhani et al., 2019). However, MS does not predispose to colitis (IL-10 KO model) or enteric infection by *Salmonella* or *Listeria* (Riba et al., 2018).

We hypothesize that intestinal barrier dysfunction, which precedes the onset of metabolic phenotype, is triggering metabolic disorder in our model.

The role of intestinal barrier in metabolic disorder is highly discussed. Some consider intestinal barrier dysfunction as a consequence, other as a cause of metabolic disorder. Cani et

al. showed that increased endotoxemia (bacterial products in the blood) leads to metabolic disorder (Cani et al., 2007). In *db/db* and *ob/ob* mice increased intestinal permeability is observed (Brun et al., 2007). Thaïss et al. argue that hyperglycemia is triggering intestinal barrier dysfunction in diverse models (STZ induced, *ob/ob*, *db/db*, ...) (Thaïss et al., 2018). We think, that intestinal barrier dysfunction in male mice is triggering intestinal and systemic low-grade inflammation responsible for a loss of insulin sensitivity. Indeed, it has been shown, that especially TNF α is able to interfere with insulin signaling. This interference can diminish insulin signaling and its action in the cell. Therefore TNF α is a potent inducer of insulin resistance (Feinstein et al., 1993; Hotamisligil et al., 1996, 1993). Furthermore, microbiota dysbiosis has been described to be an important player in the pathogenesis of metabolic disorder. Indeed, metabolic disorder such as obesity and T2D are associated with gut dysbiosis (Forslund et al., 2015; Ley et al., 2005). Metabolic disorder are also associated with intestinal hyperpermeability (Cani et al., 2008) and modified intestinal immune response (Garidou et al., 2015; Mohammed et al., 2012).

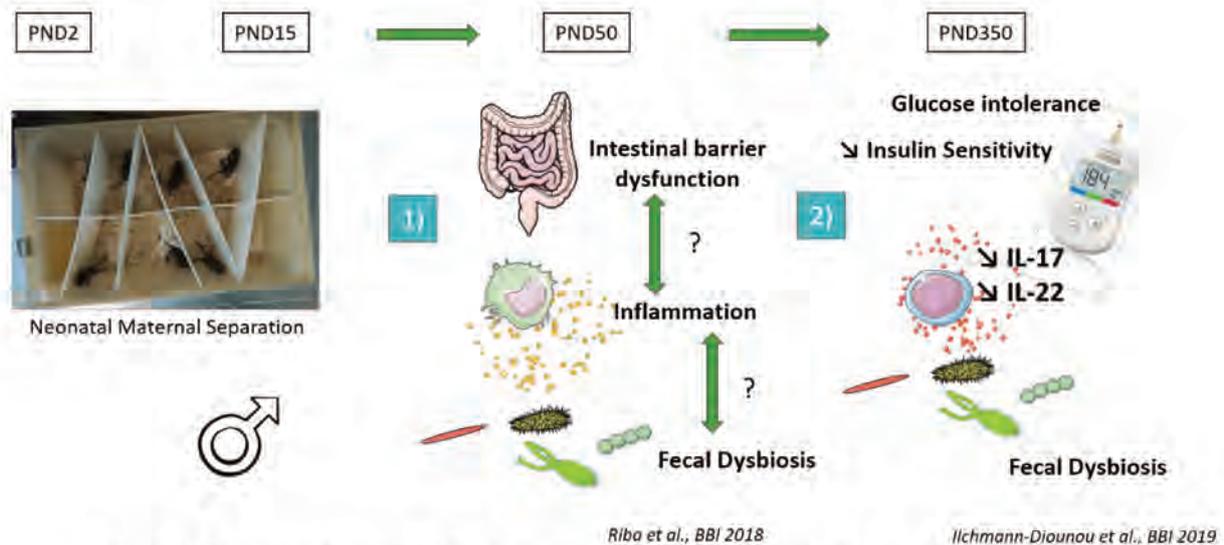


Figure 28 Temporal aspects of the phenotype induced by maternal separation in male mice.

Interestingly, there is also a link between metabolic disorder and intestinal bowel syndrome (IBS). MS has been used for a long time as a model for IBS. Here we demonstrated that MS induced metabolic disorder in aging rodents. This could seem surprising but epidemiological data are supporting the link between IBS and metabolic disorder. Indeed, gastrointestinal complications have been described in obesity (Camilleri et al., 2017) and in T2D (Badedi et al., 2016). Interestingly, in obese patients intestinal symptoms similar to IBS are documented (abdominal pain, diarrhea, fecal urgency) (Pugliese et al., 2019). In a Swedish cohort, obesity was associated with IBS and abdominal pain (Aro et al., 2005). Indeed, obese

patients have increased risk to develop IBS in comparison to general population (Svedberg et al., 2002). These observations underline the importance of intestine in obesity. On the other hand there are epidemiological studies describing that IBS is a risk factor for following metabolic disorder (Badedi et al., 2016; Gulcan et al., 2009; Guo et al., 2014). A common characteristic in IBS and metabolic disorder is low-grade inflammation. This could also be the underlying factor linking one to another.

To conclude on the MS male study at PND350, this work provides indications that a defective intestinal barrier could be at the origin of later metabolic disease.

In our study with female mice (*cf.* III. Part Results, Third Result), we also observed intestinal barrier dysfunction in young adults. Even though intestinal permeability is not modified by MS, there is a decrease of antimicrobial defense in the intestine; Indeed, lysozyme concentrations in Paneth cells were decreased as well as fecal antimicrobial activity. Additionally blooming of *E. coli* was observed (Riba et al., 2017). Lysozyme activity at PND15 still needs to be assessed. In aging female mice, we observed a decrease of insulin secretion in response to glucose stimulus and a slight tendency for decreased β -cell surface (*cf.* III. Part Results, Third Result). Increased susceptibility to autoimmune diabetes still need to be confirmed.

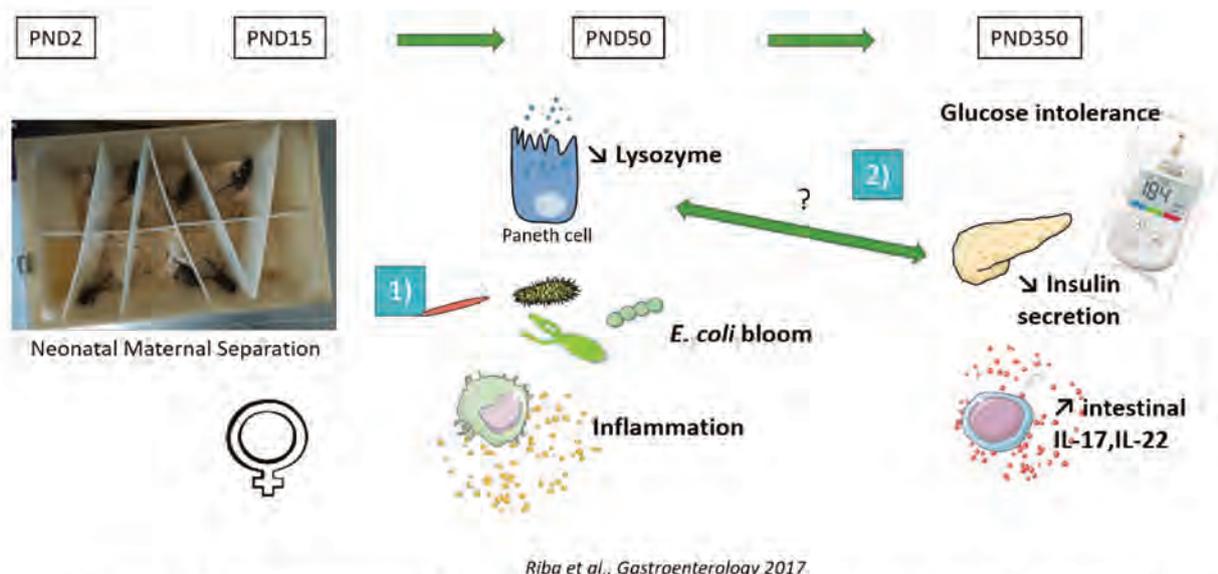


Figure 29 Temporal aspects of the phenotype induced by maternal separation in female mice.

The role of intestinal permeability in T1D is discussed. There is still no consensus if intestinal hyperpermeability is cause or consequence of disease (Buckner and Greenbaum, 2017; Harbison et al., 2019; Odenwald and Turner, 2013). In rodents, intestinal

hyperpermeability was observed before the onset of disease in biobreeding diabetic prone (BBDP) rats (Meddings et al., 1999). Additionally, tight junction proteins were modified in BBDP (Neu et al., 2005). In epidemiological studies, different results have been observed. Bosi et al. demonstrated that intestinal hyperpermeability, as measured *in vivo* by lactulose/mannitol (L/M) test occurs before disease onset (Bosi et al., 2006), others found increased *in vivo* cellobiose/M intestinal permeability in established but uncomplicated T1D (Carratù et al., 1999). While Kuitunen et al. did not observe increased intestinal permeability (L/M) in T1D patients (Kuitunen et al., 2002). Relatives of T1D patients had intermediate *in vivo* L/M intestinal permeability between T1D and control (Sapone et al., 2006). In rats, reversion of intestinal hyperpermeability by treatment with a zonulin 1 (intestinal homolog of an *Vibrio cholerae* enterotoxin that reversibly increase intestinal permeability) inhibitor ameliorates T1D manifestation in rat model (Watts et al., 2005). Our study, addressing intestinal permeability in T1D in non-obese diabetic (NOD) mice compared to non-diabetic NOD mice by *in vivo* and *ex vivo* measurements, showed important intestinal modifications during T1D in NOD/ShiltJ mice. In the NOD mice model, we observed increased *in vivo* intestinal permeability (FD4) but decreased *ex vivo* paracellular permeability associated with striking intestinal modifications only after the onset of diabetes. We excluded an *ex vivo* intestinal hyper-permeability expressed in cm/s in diabetic mice but agreed with a higher *in vivo* exposure to luminal content.

Due to the proximity between pancreas and duodenum, bacterial translocation from the intestine *via* the pancreatic duct towards pancreas could be hypothesized. 40% of long-term T1D patients had periductal fibrosis (Meier et al., 2005), which could be an indicator for persisting duct inflammation, possibly induced by bacterial infection. Microbial translocation in pancreatic lymph nodes activates NOD2 and IL-17 production in pancreatic lymph nodes and pancreas contributes to T1D development (de Goffau et al., 2013). Indeed, Korsgren et al. instilled different bacteria into the pancreatic duct. This leads to consecutive islet inflammation and T1D. Different bacteria were tested, among them *E. coli*, which exhibits the most severe pancreatic inflammation in comparison to *S. aureus*, *E. faecalis*, *α -Streptococcus* (Korsgren et al., 2012). This is of particular interest since *E. coli* has been increased in fecal microbiota of PND50 female MS mice due to defect of AMP in Paneth cells of small intestine. AMP expression is not uniquely found in Paneth cells, also neutrophils and epithelial cells can express and secrete AMPs. It is interesting to consider that antimicrobial peptides expression has also been found in pancreas. Indeed, the AMP repertoire seems to be diverse in pancreas, however in human pancreas their expression is not yet fully known (Stenwall et al., 2019). The presence

of CRAMP in the pancreas has been shown to protect mice from autoimmune diabetes (Sun et al., 2015). Stenwall et al. analysed pancreas of healthy control and one T1D patient. They found differentially expressed AMP in healthy vs the patient. AMP were generally less present in the patient than in control, even in the inflamed parts of pancreas of the patients, where AMP expression was higher than in non-inflamed parts (Stenwall et al., 2019). It could be possible, that early life stress is not only decreasing intestinal AMP activity, but also AMP activity in pancreas leading to pancreatic inflammation, which could end up in destruction of insulin-producing β -cells. To answer this question we plan to assess the presence of the AMP CRAMP in pancreas and small intestine of MS and control mice. Indeed, it is of particular interest that CRAMP is only expressed in small intestine from birth to PND15 corresponding to the timeframe of MS protocol. However, in our model in C3H/HeN mice the effect of MS on pancreas were modest so it would be also interesting to perform those analyses in a genetically predisposed mouse model.

In conclusion, the work of my PhD project highlights the role of intestinal barrier dysfunction in extra-intestinal, namely metabolic and autoimmune, diseases. The challenging hypothesis of intestinal barrier dysfunction and bacterial translocation preceding disease onset and its causal role in the pathogenesis of those diseases still need to be assessed in interventional studies.

THE ROLE OF LOW-GRADE INFLAMMATION IN AGING - INFLAMMAGING

During my PhD thesis, we showed that neonatal maternal separation (MS) leads to metabolic disorder in aging male mice. It is interesting to consider that metabolic disorder did not appear at an earlier time point. Indeed, “aging is a ubiquitous complex phenomenon that results from environmental, stochastic, genetic, and epigenetic events in different cell types and tissues and their interactions throughout life” (Franceschi and Campisi, 2014). Aging is characterized by decreased capacity to react to diverse stressors and as a consequence, gradually increasing pro-inflammatory status, a phenomenon called “inflammaging”. Chronic low-grade inflammation establishes (Franceschi et al., 2006), resilience decreases. These are risk factors to develop metabolic but also other non-communicable diseases (NCDs).

There are multiple propositions for the origins of the inflammaging process in aging individuals (for review (Franceschi and Campisi, 2014)). Damaged macromolecules and cell debris (self) can accumulate due to inadequate elimination. Harmful metabolites, products and fragments from bacteria or other sources (non-self) leak inside the organism due to inappropriate barrier function. Indeed, intestinal barrier dysfunction have been described in aging (Man et al., 2015). Cellular senescence, which is arrest of cell proliferation, is also playing a role in aging. In elderly, there is increased activation of the coagulation system (Hager et al., 1989) which can be considered as part of the immune system due to its strong interaction with the last, underlining a disturbed immune system in the elderly. Another parameter favoring inflammaging are the changes in the immune system, also called immune senescence. Indeed, there have been multiple observations on immune system in elderly. Some authors propose that the adaptive immune response undergoes great changes and declines, whereas the innate immune response seems to stay preserved and becomes more reactive (Franceschi et al., 2000).

Thanks to previous work and my PhD project, we had the possibility to follow several markers of the immune system at different time points during our protocol in C3H/HeN male mice, namely in early life (PND7, PND15, PN21), in young adulthood (PND50) adult (PND100, PND150) and in aging (PND350). Due to experimental reasons, not all data are available for every time point.

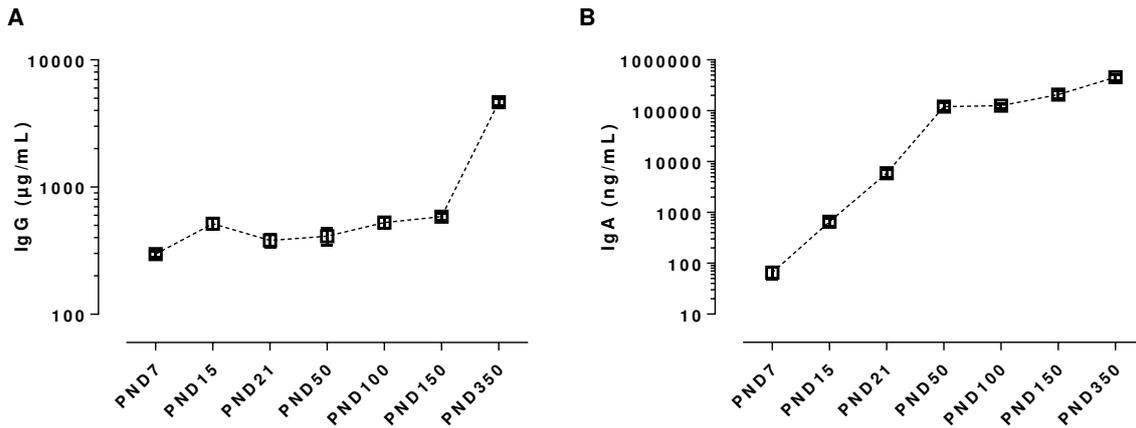


Figure 30 Plasmatic immunoglobulin concentrations measured by ELISA in C3H/HeN male mice at post-natal day (PND) 7, 15, 21, 50, 100, 150 and 350. (A) IgG ($\mu\text{g/mL}$). (B) IgA (ng/mL)

In C3H/HeN mice, IgG and IgA plasmatic concentrations increase in time (**Figure 30**), whereas IgG increased sharply in aging mice (PND350, **Figure 30A**). Paganelli et al. observed similar phenomena in human. They studied a cohort, including males and females, in the age range from 23 years to 106 years. They showed that plasmatic IgG and IgA increased with aging (Paganelli et al., 2008).

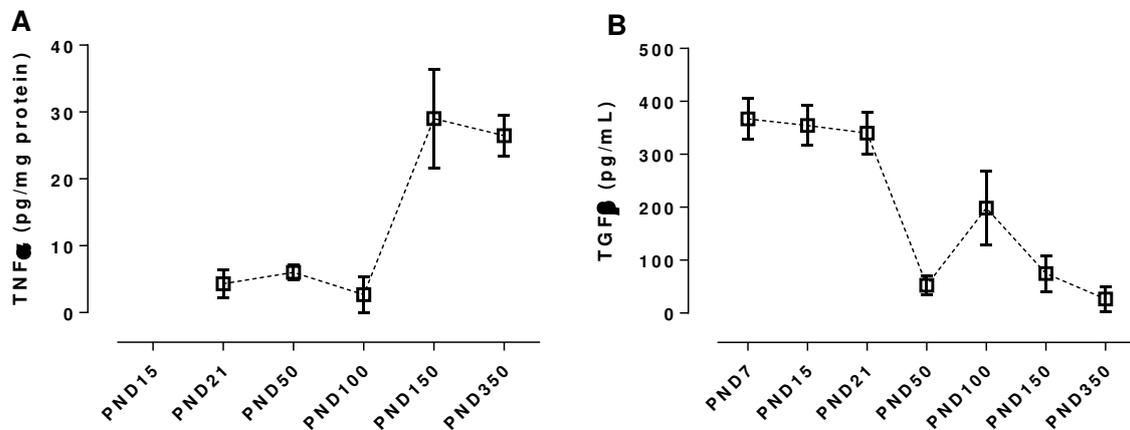


Figure 31 Cytokine concentrations measured by ELISA in C3H/HeN male mice at post-natal day (PND) 7, 15, 21, 50, 100, 150 and 350. (A) TNF α concentrations (pg/mg protein) in lysate of small intestine. (B) TGF β secretion (pg/mL) in response to anti-CD3/CD28 (T cell receptor) stimulation in primary culture of splenocytes.

Cytokine secretion during lifetime is visualized in **Figure 31**. We observed cytokine concentrations in SI. TNF α concentrations increased with aging (**Figure 31A**). These results are consistent with literature. Thevaranjan et al. identified increased TNF α as driving force for decreased killing capacity of macrophages from old vs young mice (Thevaranjan et al., 2017). In human, stimulated secretion of IL-6, IL-1 β , TNF α was increased in PBMC of old (mean age

80.2) vs young (mean age 26.8) participants. There were no differences for $\text{IFN}\gamma$ secretion (Fagiolo et al., 1993).

We analyzed several cytokines of adaptive immune response at systemic level (spleen), as for example pro-inflammatory and anti-inflammatory, like $\text{TGF}\beta$ (**Figure 31B**). $\text{TGF}\beta$ secretion constantly decreased in time and reached lowest levels in aging mice. These are indications that adaptive immune response seems to decrease in aging. Indeed, the immune system in the young is characterized by strong Th2 response and secretion of the anti-inflammatory cytokine $\text{TGF}\beta$ in order to favor entry of luminal antigens for immune priming and establishment of tolerance (Adkins et al., 2004). Even though in physiological conditions $\text{TGF}\beta$ seem to decrease with age, it is interesting to note that the opposite is observed in pathologies. In human, increased $\text{TGF}\beta$ is described in aging and in numerous age-related pathologies, as for example osteoarthritis (Kriegelstein et al., 2012). In healthy state, $\text{TGF}\beta$ is necessary for maintenance of articulate cartilage, in the case of osteoarthritis decrease of $\text{TGF}\beta$ -receptor expression and modified $\text{TGF}\beta$ signaling has been described (van Caam et al., 2016). These observations highlight that not only $\text{TGF}\beta$ concentration could be affected during aging but also underlying mechanisms.

Aging is also associated with intestinal barrier dysfunctions. Indeed, with aging intestinal permeability increases and innate immune system is modified in mice (Thevaranjan et al., 2017) and human (Man et al., 2015). Intestinal IL-6 (Man et al., 2015) and $\text{TNF}\alpha$ (Thevaranjan et al., 2017) secretion are increased in aging subjects. Thevaranjan et al. showed that aging is associated with microbiota dysbiosis, which is triggering age-associated inflammation. However, both were interdependent, since microbiota dysbiosis could be reversed by treating increased $\text{TNF}\alpha$ (Thevaranjan et al., 2017). They concluded that “age-associated inflammation and microbial dysbiosis drive intestinal permeability and translocation of bacterial components, further fueling inflammation and impairing cellular antibacterial function” (Thevaranjan et al., 2017). Those results are underlining the potent role of inflammation and intestinal barrier dysfunction in aging.

Until here, I demonstrate the effects of aging on immune system and intestinal barrier in C3H/HeN mice. However, this does not explain why we observe differences between MS and control mice. Why do MS mice develop metabolic disorder with aging but not control?

Franceschi et al. propose that inflammaging alone is not sufficient to induce age-related diseases. Low-grade inflammation in aging leads to higher susceptibility to disorders, the

authors called this “first hit”. However, to develop disease a “second hit” is necessary. According to Franceschi et al. this could be genetically predisposition (Franceschi et al., 2006), but I would extend the term “second hit” by defining it as an additional risk factor like MS.

Indeed, MS mice experienced an important insult in early life – the separation from their dam, which led already in young mice to modifications of immune system and intestinal barrier. According to the theory of embodiment, this insult can leave an imprinting on the organism which lead later to higher disease susceptibility. The cost of the adaption to early life stress in MS male mice is reflected by early (PND50) development of low-grade inflammation (Riba et al., 2018). As young adult, MS mice already faced the symptoms of irritable bowel syndrome (Riba et al., 2018). These modifications can be resumed as wear and tear on the body – which try to find new homeostasis (Fava et al., 2019). In aging (PND350) male mice face higher low-grade inflammation compared to control (Ilchmann-Diounou et al., 2019). Indeed, at PND350 protective IL-17, IL-22 and IL-10 responses were decreased and pro-inflammatory TNF α secretion increased in SI.

To conclude, our work confirmed that aging is associated with profound changes in the immune system and higher susceptibility to disease. Indeed, metabolic disorder only appear at PND350 in our model.

THE MODEL OF MATERNAL SEPARATION IS A USEFUL MODEL TO DECIPHER ETIOLOGY OF METABOLIC DISORDERS

During my PhD thesis, we showed that neonatal maternal separation (MS) leads to metabolic disorder in aging (PND350) male mice without any dietary challenge. Metabolic disorder was characterized by fasted hyperglycemia, glucose intolerance and insulin resistance without obesity. Additionally, we observed microbiota dysbiosis, intestinal and systemic immune alterations and increased humoral immune response against microbiota (Ilchmann-Diounou et al., 2019).

Interestingly, low-grade inflammation was already observed in small intestine and spleen in our model at earlier time point (PND50) (Riba et al., 2018) and persists at PND350 with increased LPS-induced TNF α secretion in siLP and spleen suggesting an imprinting of early life. Additionally, intestinal barrier dysfunction has been observed at PND50, namely decreased lysozyme activity, intestinal hyperpermeability and microbiota dysbiosis (Riba et al., 2018).

We hypothesize that weakened intestinal barrier at PND50 induces persistent low-grade inflammation that precedes metabolic disorder. Until today, there is no consensus among scientists if low-grade inflammation is cause or consequence of metabolic disorder.

Arguments in favor of low-grade inflammation as a trigger are inflammaging process (treated in the previous chapter) and experiments with anti-inflammatory treatments in metabolic diseases. In 1957, Reid and colleagues demonstrated that aspirin (salicylates), an anti-inflammatory treatment decreased fasting blood glucose levels in overweight and lean diabetic patients (Reid, 1957). Later on, studies indentified I κ B/NF κ B axis as the molecular target of hypoglycemic effects of salicylates (Shoelson et al., 2003; Yuan et al., 2001).

Our data suggest that defect of intestinal barrier functions (intestinal hyperpermeability and defect of Paneth cells associated with microbiota dysbiosis) and low-grade inflammation observed in PND50 male mice submitted to MS (Riba et al., 2018) precedes the onset of glucose intolerance and insulin resistance observed in PND350 mice. This observation is in accordance with studies suggesting that high-fat diet impairs intestinal barrier (intestinal hyperpermeability and defect of Paneth cells) and triggers low-grade inflammation before the onset of adverse metabolic consequences (for review, (Araújo et al., 2017)). Furthermore, in mouse model of HFD-induced intestinal hyperpermeability associated with an increase of HOMA index (a

measure for insulin resistance), restoring intestinal permeability by fish oil treatment or resolving D1 did not improve HOMA index (Lam et al., 2015), suggesting that correcting intestinal permeability is not sufficient to ameliorate metabolic status. One could hypothesize that, past intestinal hyperpermeability have been already responsible for the establishment of low-grade inflammation. Correcting hyperpermeability at late time point when metabolic disorder is established may be too late to correct immune modifications. In our model, we observe corrected intestinal permeability at PND350, however, metabolic disorder only appeared at this time point. MS increased humoral immune response against-*E. coli* in plasma from PND50 and this signature persists until PND350. Interestingly, IgG against specific bacterial antigens were increased in diabetic patients (Mohammed et al., 2012).

These results are encouraging to set up an experimental model to decipher whether metabolic disorder are a consequence or a cause of an ongoing low-grade inflammation, a question still under debate in metabolic disorder and particularly in T2D. Indeed, since metabolism and inflammation are closely related and regulate one another (as previously discussed), in established metabolic disorder with inflammation it is difficult to discriminate which one – inflammation or metabolic disorder – precedes the other. The neonatal maternal separation paradigm seems an appropriate model in order to answer this question and to evaluate the mechanisms linking intestinal barrier, immune changes, low-grade inflammation and glucose metabolism disorders, which seem to appear in a distinct time frame in this model. Using the model of neonatal maternal separation to study metabolic disorder may contribute to a better understanding of the relationship between immunology, intestinal barrier (including microbiota) and metabolism. In the following, I am suggesting some interventional studies, which could be useful to respond to the question.

Since metabolic disorder in our model appear late, it would be interesting to precipitate or accelerate the establishment of metabolic disorder, by a dietary challenge. Indeed, our results are obtained under normal diet. High-fat high-sugar diet (HFHSD) could be a useful tool, since it is described to induce rapidly metabolic disorder.

In a first step, one has to validate that HFHSD induces exacerbated metabolic disorder in MS mice compared to control. Indeed, preliminary results in our laboratory showed that HFHSD induces in MS male mice greater weight gain compared to control under HFHSD diet (**Figure 32**). Besides, food intake were similar between control under HFHSD and MS under HFHSD.

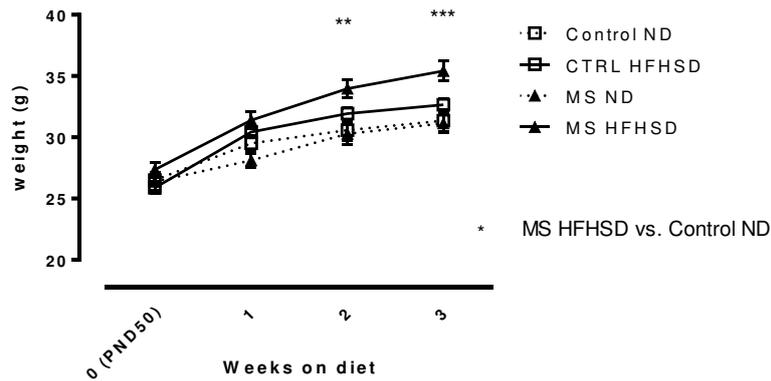


Figure 32 Preliminary data Body weight (g) from maternal separated (MS) and control C3H/HeN male mice under high-fat high-sugar diet (HFHSD) or normal diet (ND) at post-natal day (PND) 50 (start of HFHSD) and 1, 2, and 3 weeks after HFHSD. Asterisk is indicating statistic difference between MS mice under HFHSD and control mice under normal diet, two-way ANOVA, Sidak's multiple comparison test, ** $p < 0.01$, *** $p < 0.001$, $n = 7-10$ mice.

In a second step, numerous interventional studies are possible to assess the role of

- Intestinal barrier
 - Intestinal barrier correction (decrease of inflammation and intestinal hyperpermeability induced by MS) *via* pre- and probiotic treatment, before HFHSD challenge.
 - Intestinal permeability correction (decrease of intestinal hyperpermeability induced by MS) by ML-7, a MLCK-inhibitor (Moussaoui et al., 2014; Rincel et al., 2019) or zonulin inhibitor (Fasano, 2011), before HFHSD challenge
- Immune response
 - Decrease of inflammation induced by MS by non-steroidal anti-inflammatory treatment, before HFHSD challenge.

We hope that by reducing inflammation and/or improving intestinal barrier function we will prevent MS-induced glucose intolerance and increased gain weight observed under HFHSD diet compared to control on the same diet. Those studies will confirm the role of the intestinal barrier dysfunction and inflammation in the onset of metabolic disease.

THE EFFECTS OF MATERNAL SEPARATION AFFIRM THE IMPORTANCE OF NEONATAL WINDOW AND THE DOHAD CONCEPT

This PhD project underlined the early and long-term effects of early life adverse events, namely neonatal maternal separation in the development of different non-communicable diseases.

The most significant effect we observed all along the MS model is the induction of modified humoral immune response against luminal content in plasma of male and female. This effect appears already in early life (PND15). Indeed, at PND15 IgG against commensal bacteria (*E. coli*) is decreased in male, but not in female (**Figure 33A**) and specific humoral IgG immune response against food is significantly increased (**Figure 33B**) in both sexes.

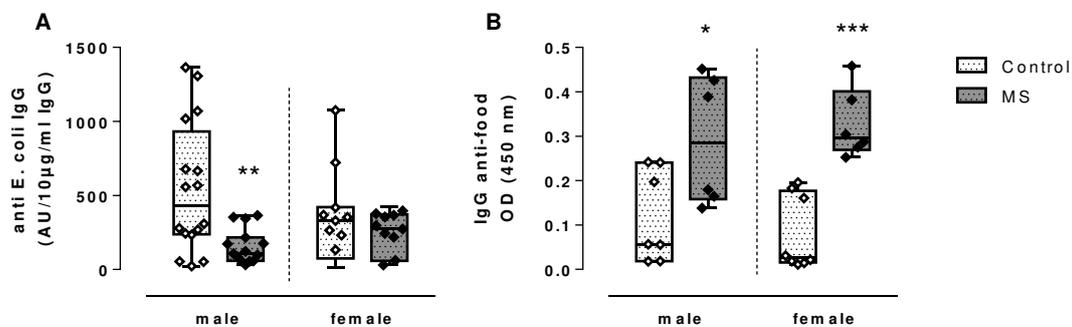


Figure 33 Specific humoral immune response measured by ELISA at post-natal day (PND) 15 in plasma of male and female C3H/HeN maternal separated (MS) and control pups. **(A)** Specific IgG against microbiota (*E.coli* lysate) (arbitrary unit (AU) of standard sample/10 µg/mL IgG). **(B)** Specific IgG against hydrosoluble fraction of food (optical density (OD) at 450 nm), Student’s unpaired t-test, * p<0.05, **p<0.01.

Interestingly, the specific immune response against luminal antigens is reversed in MS model in time. Indeed, here we show that MS reduces IgG against *E. coli* in pups. However, in MS young adults (Riba et al., 2018) and aging mice (Ilchmann-Diounou et al., 2019) IgG against commensal microbiota is increased. In contrast, IgG against food is increased in MS pups and similar in young adults (Riba et al., 2018) and aging mice (**Figure 34**).

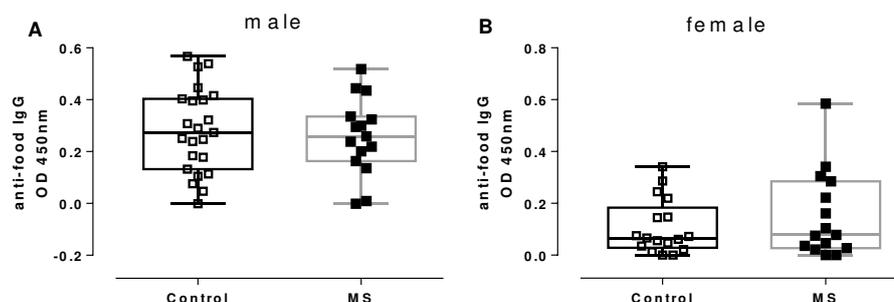


Figure 34 IgG against hydrosoluble fraction of food in plasma measured by ELISA (optical density (OD) at 450 nm) at post-natal day (PND) 350 **(A)** of male and **(B)** female C3H/HeN mice.

In summary, in pups, humoral immune response against microbiota is delayed by MS, whereas MS pups are more sensitive to food antigens. Modified immune response towards luminal antigens could also be due to different microbiota composition. Indeed, Zeng et al. identified the potential of commensal gram-negative bacteria to induce systemic specific IgG response, which was protective in later infection (Zeng et al., 2016). The increased reaction to food antigens could perhaps be explained by the fact that MS pups start earlier to introduce solid food in their nutrition than control pups. One can hypothesize that this could be due to the disturbed relationship with their dam. To answer this question, it would be interesting to observe feeding behavior in MS neonates and to study microbiota changes in early life. In contrast, with potentially advanced solid food introduction, we observe in our model in male a retarded gut closure, since intestinal permeability stay increased until PND50. The combination of advanced introduction of solid food and concomitant microbiota changes with delayed gut closure could be threatening, since more potential dangerous molecules could pass the intestinal barrier and reach the systemic circulation. However, in female there is no delayed gut closure but decreased lysozyme activity and intestinal low-grade inflammation, indicating perturbation of intestinal physiology.

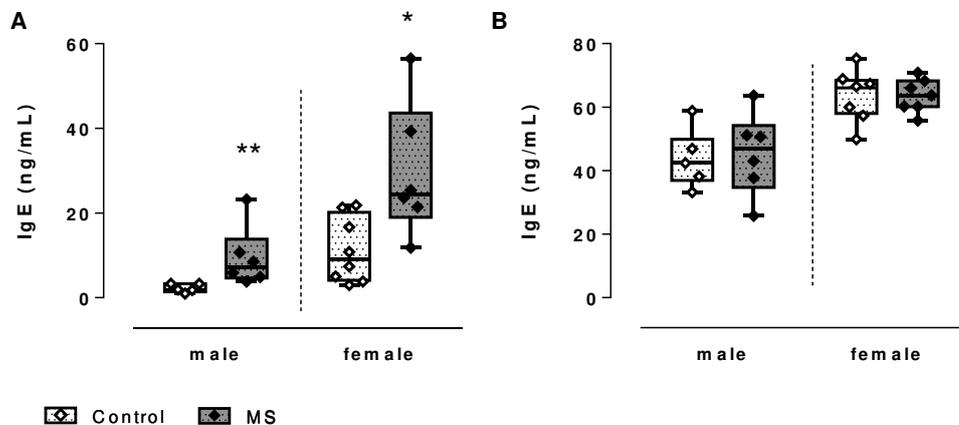


Figure 35 Plasma IgE concentrations (ng/mL) measured by ELISA in maternal separated (MS) and control C3H/HeN pups. **(A)** post-natal day (PND) 15. **(B)** PND21. Student's t-test, * $p < 0.05$, ** $p < 0.01$.

IgE concentrations were significantly increased at PND15 in male and female mice submitted to MS (**Figure 35A**). This effect was transient and already resolved at PND21 (**Figure 35B**). However, since pups are, during the neonatal period, immunological dependent on their dam it would be necessary to analyze dams milk and assess Ig content. Indeed, immunoglobulins are delivered from dam to pup by milk, which provide protection from infection during the first days of life through passive immunity. Additionally, transfer of Ig from lumen into circulation is possible via Fc receptors in mice (Van de Perre, 2003). IgE is

playing a role in allergy. Indeed, there are IgE-mediated food allergies (Berin, 2015). Interestingly, mice with low diversity microbiota as well as germ-free (GF) mice have elevated IgE concentrations in plasma. The increased IgE concentration lead to anaphylactic reactions in GF mice This deleterious outcome can be avoided by colonization with high diversity microbiota in early life (Cahenzli et al., 2013). It would be interesting to analyze microbiota in early life in the MS model. Perhaps MS leads to decreased gut microbial diversity. Additionally, it would be intriguing to set up food allergy experiments, to test if the observed effect on immunoglobulin repertoire has significant consequences on the allergy susceptibility. However, the food allergy experiment must be carried out in early life, since the observations we made in IgE and IgG against food resolve in time, thus highlighting the presence of an early critical window of opportunity.

In conclusion, our work shows that an insult in early life can have long-lasting consequences on intestinal barrier and immune system. Depending on the time the effect of MS were analyzed, different disease susceptibilities have been highlighted. At PND350 metabolic disorder, at PND50 IBS (Riba et al., 2018) and at PND15 probably food allergy. Thus MS model is highlighting that early life adverse events can have multiple consequences on physiology and as a consequence increasing overall disease susceptibility. Hence, this work supports the concept of DOHaD and the notion of a neonatal window of opportunity.

MS INDUCES SEXUAL DIMORPHISM

Interestingly in our work, we observe a MS-induced sexual dimorphism at all ages. Indeed, already at the age of 15 days male and female mice were different regarding immune response. One example is the increase in IgE concentrations in female compared to male in both control and MS group (**Figure 36**). These observations are underlining early onset of sexual dimorphism in the control and MS group.

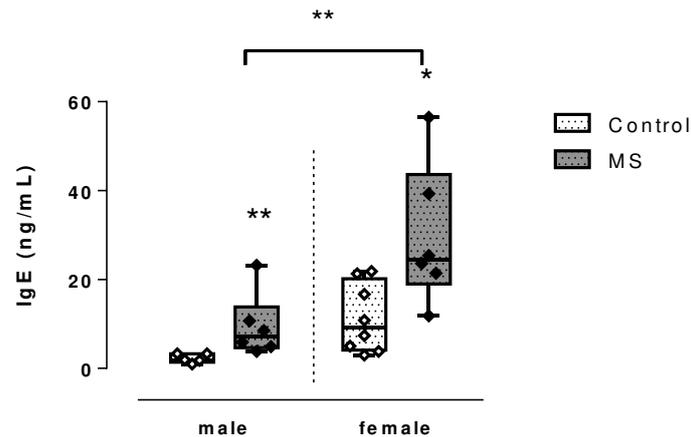


Figure 36 Plasma IgE concentrations (ng/mL) measured by ELISA in C3H/HeN maternal separated (MS) and control pups at post-natal day (PND) 15, two-way ANOVA, Sidak's multiple comparison test.

Table 2 Sexual dimorphism in MS-induced phenotype at PND350

	Male	Female
<i>Glucose tolerance</i>	Glucose intolerant	Glucose intolerant
<i>Insulin sensitivity</i>	Loss of insulin sensitivity	No change of insulin sensitivity by MS
<i>Insulin secretion</i>	No change of insulin secretion	Decreased insulin secretion in response to glucose stimulus
<i>Intestinal innate immune response</i>	Increased TNF α secretion in response to LPS stimulation	Increased basal and LPS-stimulated TNF α secretion
<i>Intestinal adaptive immune response</i>	Decreased IL-17, IL-22 and IL-10 secretion in response to TcR stimulation	Increased IL-17 and IL-22 secretion in response to TcR stimulation
<i>Systemic innate immune response</i>	Increased TNF α secretion in response to LPS stimulation	No modification by MS
<i>Systemic adaptive immune response</i>	Increased IL-17 secretion in response to TcR stimulation	Increased IFN γ secretion in response to TcR stimulation
<i>Humoral immune response</i>	Decreased fecal IgG concentrations, increased plasmatic IgG specific for microbiota	No modification by MS

The different results observed in male and female at PND350 are summarized in **Table 2**. Interestingly, our observations in aging mice are similar to epidemiological observations highlighting diseases susceptibility depending on sex. Male MS mice develop classical metabolic disorder with insulin resistance, while we hypothesize that female MS mice develop metabolic disorder with autoimmune characteristics. Elderly men developed more often metabolic syndrome than female. However, today in the United States there are more women with metabolic diseases due to faster increase in obesity in women (Beigh and Jain, 2012). Similar data were obtained in other countries, as for example Russia and Korea. However, women suffering from metabolic diseases in comparison to men with metabolic diseases have higher body weight but lower plasmatic HDL, suggesting that woman need higher degree of obesity to reach the same metabolic disturbance than man (Dallongeville et al., 2004; Williams et al., 2003). Additionally, women suffer less from metabolic disorder associated complications, as for example coronary heart disease. This is principally due to the more favorable fat distribution in women (Regitz-Zagrosek et al., 2006). Additionally, men have higher insulin resistance than woman, associated with higher hepatic and visceral adipose tissue in men (Geer and Shen, 2009). These data are matching with our results, since male MS mice developed metabolic disorder, namely glucose intolerance and decreased insulin sensitivity without obesity.

Besides metabolic disease susceptibility, there are various other diseases where male and female have different susceptibility.

In human, a great sexual dimorphism is seen in the susceptibility to autoimmune diseases (AD). Female are more prone to develop autoimmune disease. Indeed, about 45 of 80 identified auto-immune diseases are female-biased (Hayter and Cook, 2012). Since the onset of most AD is around 40 years of age, sexual hormones have been rapidly identified as the trigger of differences in susceptibility. However, there are also sex-biased AD with pediatric onset, long before puberty, as for example juvenile idiopathic arthritis (Ravelli et al., 1998).

These data are consistent with our observations. It seems that female MS mice develop metabolic disorder with autoimmune characteristics, namely loss of insulin secretion that still need to be confirm by results of MS on diabetes incidence on NOD female mice.

The male and female immune response are different. Female develop robust adaptive immune response and are as a consequences more protected from infection than males, which develop more easily inflammation (Fairweather et al., 2008; Klein and Flanagan, 2016). The

differences in the immune system have been finely documented and largely attributed to sexual hormones (Oertelt-Prigione, 2012). Data on pre-pubertal immune system is sparse or lacking (Chiaroni-Clarke et al., 2016).

There are also differences observed in the susceptibility to mental and psychological disease. Indeed, women develop twice-often depression in comparison to men (Karger, 2014). The ratio of boys:girls diagnosed autism spectrum disorder is 3:1 (Loomes et al., 2017).

The stress response and the HPA axis response differ between male and female. Female sex hormones attenuate the sympathoadrenal and HPA responsiveness, leading to slowly cortisol feedback on the brain and less or delayed containment of the stress response (Verma et al., 2011). Studies in female control, ovariectomized and oestrogen and progesterone-replaced rats showed that ovarian steroids influence the HPA axis (Carey et al., 1995). Other studies argue that the observed HPA differences in male and female emerge from the organizational effects of gonadal steroids during early brain development (Patchev and Almeida, 1998). However, in human obtained data are conflicting. In women, cortisol concentrations were not correlated to menstrual cycle (Abplanalp et al., 1977).

Additionally, glucocorticoid receptors (GR) are differentially expressed in male and female. On most leucocytes, GR are more expressed in male compared to female (Lu et al., 2017). This could be one reason for modified immune response in male and female.

What could be the reasons for sexual dimorphism apart of sex hormones? Are there other explications for sexual dimorphisms?

Even if sexual hormones are similar in pre-pubertal stage, sex hormone receptor expression could be different. Indeed, a study in sheep showed that estrogen receptor (ER) but not androgen receptor (AR) were differentially expressed in male and female sheep already during gestation (Reddy et al., 2014). In rats, dimorphic ER expression has been found in the early neonatal period (Orikasa et al., 2002). ER α and ER β are expressed in a variety of tissues and cells. They are present in intestinal tissue (Kawano et al., 2004), whereas ER β is expressed twice times more in female colonic epithelium compared to male (Campbell-Thompson et al., 2001; Thomas et al., 1993). They can also be found on numerous immune cells (Grimaldi et al., 2002; Phiel et al., 2005).

Other mechanisms, which lead to sexual dimorphism, could be due to genetics. X-chromosome silencing or epigenetic mechanisms (Chiaroni-Clarke et al., 2016).

In human, sex-bias can also be due to different exposure between genders due to occupation, however, in our study, male and female mice were living in the same environment and no social gender-effect can be accused.

There is no standard reason to choose one sex or another in animal studies for general purposes. Indeed, historically female animals were often excluded due to the hormonal changes during the sexual cycle. Researchers supposed that these hormonal changes in female would induce greater variability in data and a need for higher number of groups in the experiments to find statistical significance (Shansky, 2019). This lead to a great bias in several disciplines of biomedical research (Beery and Zucker, 2011). However, there were no scientific study proofing this assumption. Even more surprisingly, a meta-analysis of variability did not find that female data are more variable than male (Becker et al., 2016) but demonstrated in the contrary that testosterone levels in male subjects have a great variability depending on their social position (dominant or subordinate) in the cage (Machida et al., 1981).

Carrying out preclinical studies in sex-biased experiments, can have important impact on the results and therefore adverse effects on especially women's health. Only in 2014, but luckily, the NIH changed its policy to face the sex-bias in biomedical research. Indeed, the NIH requires now that male and female cells and animals are included in preclinical studies, except of applications were use of both sexes is not justified (Clayton and Collins, 2014).

In summary, our work shows that male and female are, from young age on, biologically different and highlights sex as an important variable in biomedical studies. Our work emphasizes the need to conduct research in male and female subjects, since observed effects could be quite different and conclusions of studies carried out only in one sex could not necessarily be transposable to the other sex.

V. PART CONCLUSIONS



This PhD project identified for the first time neonatal maternal separation as a risk factor for Non-Communicable Diseases in male and female C3H/HeN mice. We observed a sexual dimorphism confirming epidemiological data: male mice developed metabolic disorder with insulin resistance, while female seem to develop autoimmune disorder. The results of our work show that sexual dimorphism is a real challenge in biomedical research and that dimorphic phenotype can appear already in early life.

Our results highlight the neonatal window as a critical period for the establishment of immune homeostasis and a life-long appropriate interplay between microbiota, immune response and metabolic functions, hence strengthening the concept of Developmental Origin of Health and Diseases (DOHaD).

In our work, we did not analyze any epigenetic signature, since it was beyond the aim of this project. Nevertheless, epigenetic regulations are potent mechanisms in the mediation of early life environment and later health outcomes. Further studies in the MS model are needed to assess the impact of early life stress on epigenetic signature and its role in the development of non-communicable diseases.

Our work underlines the role of intestinal integrity in health and disease. Indeed, intestinal modifications are observed in intestinal and extra-intestinal diseases, in some case even before the onset of disease. Hence, preserving intestinal integrity should be one of personal and medical occupations. Indeed, preservation of overall health and prevention of disease could be improved and supported in taking care of our gastro-intestinal tract at early time point. Care and treatment could be food remediation, applications of pre- and probiotics. Indeed pre- and probiotics are already used in different diseases in order to regain a healthy and stable microbiota. Dietary intervention are still the first choice of treatment in metabolic disorder. Additionally, intestinal health could also be targeted via the HPA axis by stress management.

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**Consequences of early life adverse events on the development of non-communicable diseases
in mouse models**

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Toxalim Toulouse, 19 September 2019

The concept of Developmental Origins of Health and Disease (DOHaD) highlights the importance of early life period and raises the hypothesis that Non Communicable Diseases (NCD) could find their origins in perinatal environment. Neonatal maternal separation (MS) is a stress model widely used in rodents as a paradigm of early life adverse events. In my PhD project, I aimed to investigate in aging male and female wild-type mice under normal diet the long-term effects of neonatal MS on intestinal barrier function, metabolism, immunity, auto-immunity, as well as on microbiota. My work aimed to provide experimental data to support a link between early life stress and development of metabolic or autoimmune disorders with aging.

In our first study, MS led to glucose intolerance and loss of insulin sensitivity associated with fecal dysbiosis in Post Natal Day (PND) 350 wild-type C3H/HeN male mice fed a standard diet. Fecal IgG concentrations were decreased in MS mice compared to control mice, whereas anti-*E. coli* IgG, representing humoral response toward commensal microbiota, were significantly increased in plasma of MS mice. MS significantly decreased IL-17 and IL-22 secretion in response to TcR stimulation in small intestine *lamina propria* (siLP) culture. Besides, TNF α secretion in response to LPS-stimulation was slightly increased. The same results were obtained at systemic level (spleen). For the first time, we demonstrated that early life stress alone is a risk factor for metabolic disorders development in aging wild type mice under normal diet. The result of this project gave us the opportunity to question the role of microbiota in MS-induced glucose intolerance. Fecal microbiota transfer of MS mice microbiota was not sufficient to induce glucose intolerance.

In our second study in PND350 female, MS increased IL-17 and IL-22 by siLP cells in response to TcR stimulation. TNF α secretion with and without LPS stimulation was also increased by MS. Additionally, we observed systemic low-grade inflammation. MS mice developed glucose intolerance associated with decreased insulin secretion in response to glucose stimulus. Ratio of β -cell surface to pancreas surface was slightly decreased in MS mice compared to control. This ratio positively correlated with insulin secretion induced by glucose. Taken together, the results of our study showed that MS in wild type female mice under normal diet leaves a long-lasting imprinting on immune-metabolism and pancreas homeostasis.

We compared *in vivo* and *ex vivo* intestinal permeability measurements in a model of type 1 diabetes (NOD – non-obese diabetic mice). Intestinal permeability was assessed *in vivo* by gavage and *ex vivo* in Ussing chambers with the marker FITC-Dextran 4 kDa. Surprisingly, the results of both methods were divergent. The difference between *in vivo* and *ex vivo* measurements could not be explained by altered renal excretion. Curiously, diabetic NOD mice had significantly longer small intestine than non-diabetic NOD mice and small intestine length positively correlated with intestinal permeability *in vivo*. However, there were no difference in intestinal transit time, feces humidity and histological appearance. Altogether, our results highlighted the importance to distinguish intestinal permeability, which is expressed as cm/s, measured *ex vivo*, and the notion of systemic exposition to luminal antigen, measured *in vivo*.

My PhD project shows that early life adverse events are a risk factor for NCD. Interestingly, our observations in aging mice are similar to epidemiological observations. Indeed, preliminary results suggested that female MS mice develop metabolic disorders with autoimmune characteristics but male MS mice develop classical metabolic disorders with insulin resistance. My work in MS model highlights the importance of early life in the establishment of homeostasis and comforts the concept of DOHaD.

Keywords:

Social stress, Glucose metabolism, Intestinal barrier, Immune response, Developmental origin of health and diseases (DOHaD)
