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Microenvironmental modulation of the intestinal epithelial barrier

Fagundes Rosa, Raphael

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Microenvironmental modulation of the intestinal epithelial barrier

PhD thesis

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on the authority of the
Rector Magnificus Prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

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Wednesday 16 March 2022 at 16:15 hours

by

Raphael Fagundes Rosa

born on 12 December 1991
in Santana-BA, Brazil

Promotors

Prof. K. N. Faber

Prof. G. Dijkstra

Dr. H. J. M. Harmsen

Assessment committee

Prof. J. M. van Dijl

Prof. C. Y. Ponsioen

Prof. S. P. Colgan

Paranymphs

Dr. Carmem Maia Gilardoni

MSc. Marcel A. Vieira Lara

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Summary

The gastrointestinal (GI) tract shapes the health of numerous organs and systems in an organism. Human health can be directly associated with the presence and abundance of different species of microorganisms inhabiting the GI tract, especially bacteria. Previously known as flora, these nearly thirty trillion bacteria that colonize each of our bodies are collectively named microbiota, and the majority of these bacteria are located in the large intestine.

Human cells need oxygen to survive, however, the gut lumen becomes anaerobic in the large intestine, location where most of the fermenting bacteria colonization takes place. The composition of the gut microbiota is extremely dynamic and significant changes are delimited by sex, age, dietary habits and genetic background of each individual. There are bacterial species that are directly associated with human health, including the anaerobe *Faecalibacterium prausnitzii*. On the other hand, the contents that go through our gut (i.e., digesta) may also contain harmful components, such as toxins or pathogenic bacteria. In order to provide a physical protection between the digesta and our systemic circulation, epithelial cells in our intestines are highly specialized in creating a tight barrier that allows the selective absorption of compounds from the digesta, which include carbohydrates, fatty acids, amino acids and specific proteins. Simple sugars, fatty acids and vitamins are absorbed in the small intestine, while, in the large intestine, the bacterial digestion of remaining undigestible food components generate absorbable nutrients for the host. Of special importance for human health, short-chain fatty acids (SCFAs) are produced by bacterial fermentation in the large intestinal lumen. There are several types of SCFAs, butyrate being the most well-known to play a key role in human health by regulating intestinal barrier function, cellular metabolism and exerting anti-carcinogenic effects. *F. prausnitzii* is an example of a bacterial specie that is able to ferment complex carbohydrates in order to produce butyrate, which is then absorbed by specialized intestinal epithelial cells.

Changes in the composition of the gut microbiota are linked to numerous diseases, including type-2 diabetes, colorectal cancer and inflammatory bowel disease (IBD). IBD is an umbrella term to describe Crohn's disease and ulcerative colitis. IBD is often diagnosed for the first time at a young age (below 30-years old) and is typically followed by intermittent periods of inflammation and remission of the GI tract. The active inflammation provokes abdominal pain, anemia, diarrhea, constipation and may eventually lead to surgical resection of patient's bowel. Unfortunately, IBD cannot be cured at the moment, but great attention is given to the effect of the microbiota in regulating inflammation or extending remission periods. Patients with IBD often present low levels of *F. prausnitzii* in their stool, compared to healthy individuals. The great

question that we try to unravel is how to modulate the gut milieu in order to relieve the inflammatory process these patients suffer from.

In my thesis, I aimed to unravel factors of the intestinal milieu that impact the function and health of intestinal epithelial cells. I hypothesized that specific features of the healthy or inflamed intestinal microenvironment directly impact the intestinal barrier, especially at the gene expression level. We tested the effect of dietary carbohydrates, gut bacteria, physiological gases and inflammatory mediators in intestinal epithelial Caco-2 cells, using the Human-oxygen Bacteria-anaerobe (HoxBan) *in vitro* model. With the HoxBan system, we were able to co-culture pure cultures of a single bacterial species or stool-derived bacterial communities with human intestinal (Caco-2) cells. In addition, we validated many of our findings analyzing the bacterial and metabolite composition of human stool samples, as well as gene expression levels of intestinal biopsies from patients with IBD. In order to collectively organize the findings of this theses, I divided the scientific content in two parts: gut microbiota and intestinal epithelial health (**Part I**) and hypoxia, inflammation and intestinal epithelial health (**Part II**).

In the first part of this thesis, I focused on the communication and interdependence between gut microbes and host intestinal epithelial cells. We describe that butyrate is an important energy source for the epithelium, but there are certainly other metabolites that may be used for epithelial growth and repair, such as simple sugars (**Chapter 1**). In fact, we showed that fermentation of the undigestible fiber inulin by *F. prausnitzii* produces excess fructose, a carbon and energy source that is readily available for the human colonic epithelium (**Chapter 2**). Moreover, we presented the development of the *in vitro* model HoxBan-Stool system, which allowed the coculture of live stool-derived microbial communities with intestinal epithelial cells (**Chapter 3**). In here, we showed that inulin supplementation to mixed bacterial communities increased the viability and expression of barrier protective genes in intestinal epithelial cells. The development of the HoxBan-Stool *in vitro* model allows fast-track testing of new drugs that potentially help modulating and preventing inflammatory flares in patients with inflammatory diseases of the GI tract.

For the second part of this thesis, we shifted our focus to microenvironmental regulation of intestinal epithelial cells in the context of IBD. In this part, I specifically focused on an important condition that surrounds the intestinal wall (mucosa): low oxygen or “hypoxia”. Intestinal epithelial cells form the barrier between the anaerobic gut lumen and the oxygenated intestinal mucosa, thus the oxygen supply to these cells is usually lower than their demand, in a phenomenon known as physiologic hypoxia. As a highly dynamic monolayer, intestinal epithelial cells are programmed to adapt to such low oxygen conditions, mostly due to the activation of the hypoxia-sensitive pathway that is controlled by the hypoxia-inducible factor 1- α (HIF1 α). Oxygen demand exceed supply in hypoxia, activating the HIF1 α pathway by blocking its protein

degradation, which triggers the gene expression of numerous proteins involved in metabolism, vascularization, extracellular matrix production and barrier function among others. Interestingly, inflammatory processes in the intestinal mucosa cause higher demand and lower supply of oxygen to the tissue, a process known as pathophysiologic hypoxia.

In **Chapter 4**, I reviewed what is known in literature about factors in the intestine milieu that determine the activation of the HIF1 α pathway in intestinal epithelial cells. I showed that hypoxia is a key feature of the intestinal mucosa, which strongly influences both physiologic and pathophysiologic processes in the intestinal mucosa. **Chapter 5** is dedicated to the intestinal host-microbe interaction and its implication to the activation of HIF1 α -dependent gene expression. We identified the cytokine interleukin-18 as the key messenger between exposure of intestinal epithelial cells to the gut bacterium *F. prausnitzii* and activation of HIF1 α pathway, which are both potential targets to promote mucosal healing in IBD. In **Chapter 6**, we studied the effect of iron deficiency on HIF1 α activation in the intestinal mucosa of patients with IBD, thus identifying the gene expression of the iron transporter transferrin receptor as dependent upon activation of HIF1 α pathway in the context of inflammation and iron deficiency in IBD. Finally, **Chapter 7** is dedicated to understand the impact of inflammation in the intestinal mucosal activation of HIF1 α pathway in patients with ulcerative colitis. We characterized that the intestinal mucosal inflammation triggers activation of the HIF1 α pathway towards a barrier-protective effect. Taken together, these studies point to the HIF1 α pathway as a potential target to maneuver the manifestations of IBD in patients.

In summary, the findings of this thesis demonstrate that the health of the human intestinal mucosa is directly determined by its surroundings. We showed that gut microbes, food components, iron deficiency, inflammation and hypoxia all influence aspects of the intestinal epithelial barrier that may be directly linked to mucosal health. The work in this thesis sheds light on therapeutic targets that may help patients with gut barrier-defective diseases, such as, but not exclusively, IBD.

Samenvatting

Het maag-darmkanaal is bepalend voor de gezondheid van talrijke organen en systemen in een organisme. De gezondheid van de mens kan veelal direct in verband worden gebracht met de aanwezigheid en hoeveelheid van verschillende soorten micro-organismen die zich in het maag-darmkanaal bevinden, met name bacteriën. Deze bijna dertig biljoen bacteriën die ons lichaam koloniseren staan ook wel bekend als ‘flora’ en worden gezamenlijk ook wel met de term ‘microbiota’ aangeduid, waarvan de meeste bacteriën zich in de dikke darm bevinden.

Menselijke cellen hebben zuurstof nodig om te overleven, maar het darmlumen wordt zuurstofarm (anaëroob) in de dikke darm, wat nou juist de locatie is waar de meeste kolonisatie van fermenterende bacteriën plaatsvindt. De samenstelling van de darmmicrobiota is extreem dynamisch en belangrijke veranderingen hierin worden beïnvloed door geslacht, leeftijd, voedingsgewoonten en de genetische achtergrond van het individu. Er zijn bepaalde bacteriesoorten die vaak in direct verband gebracht worden met de menselijke gezondheid, waaronder de anaërobe bacterie *Faecalibacterium prausnitzii*. Aan de andere kant kan de inhoud die door onze darm gaat (d.w.z. ons verteerde voedsel) ook schadelijke componenten bevatten, zoals toxines of ziekmakende (pathogene) bacteriën. De zogenaamde ‘epitheelcellen’ in onze darmen zijn uitermate geschikt om een fysieke bescherming te bieden tussen de spijsvertering en de bloedcirculatie. Deze cellen creëren een nauwe barrière die de selectieve opname van voedingsstoffen uit onze spijsvertering mogelijk maakt, waaronder koolhydraten, vetzuren, aminozuren en specifieke eiwitten. Eenvoudige suikers, vetzuren en vitamines worden opgenomen in de dunne darm, terwijl in de dikke darm onze bacteriën zorgen voor de vertering van de resterende, voor de mens onverteerbare voedselcomponenten, waarvan de producten vervolgens voor onze darm weer opneembaar worden. Van bijzonder belang voor de menselijke gezondheid zijn de zogenaamde korte-keten vetzuren (in het Engels ook wel afgekort als “SCFA's”) die vrijkomen bij de bacteriële fermentatie in de dikke darm. Er zijn verschillende soorten korte-keten vetzuren, waarvan butyraat het meest bekend is. Dit komt omdat butyraat een sleutelrol speelt in de menselijke darmgezondheid door o.a. het reguleren van de darmbarrierefunctie, het cellulaire metabolisme en het uitoefenen van anti-carcinogene effecten. *F. prausnitzii* is een goed voorbeeld van een bacteriesoort die complexe koolhydraten kan fermenteren om butyraat te produceren, dat vervolgens wordt opgenomen door gespecialiseerde darmepitheelcellen.

Veranderingen in de samenstelling van de darmmicrobiota worden waargenomen bij een scala aan menselijke ziekten, waaronder diabetes type 2, darmkanker en inflammatoire darmaandoeningen (in het Engels ook wel afgekort als ‘IBD’). IBD is een

verzamelnaam voor twee typen chronische darmontstekingsziekten: de ziekte van Crohn en colitis ulcerosa. IBD wordt vaak voor het eerst vastgesteld op jonge leeftijd (soms nog voor het 30^{ste} levensjaar) en wordt meestal gevolgd door zich afwisselende perioden van ontsteking en remissie (rustige ziekte) van het maag-darmkanaal. Een actieve darmontsteking bij IBD kan lichamelijke klachten geven zoals buikpijn, diarree, obstipatie evenals klachten als gevolg van bloedarmoede. IBD kan bij sommige patiënten op den duur zelfs leiden tot operatieve verwijdering van de darm. Helaas is IBD op dit moment niet te genezen, maar er wordt veel aandacht besteed aan de mogelijke rol van de microbiota bij het reguleren van ontstekingen of het verlengen van perioden van rustige ziekte. Patiënten met IBD hebben vaak lage hoeveelheden van de *F. prausnitzii* bacterie in hun ontlasting, in vergelijking tot gezonde personen. De grote vraag die we in dit kader proberen te ontrafelen, is hoe we het darmmilieu zodanig kunnen beïnvloeden dat het ontstekingsproces waaraan deze patiënten lijden onderdrukt kan worden.

In mijn proefschrift heb ik als doel gesteld om de verschillende factoren van het darmmilieu die van invloed zijn op de functie en gezondheid van darmepitheelcellen in kaart te brengen. Mijn hypothese was dat specifieke kenmerken van de gezonde of ontstoken darmmicro-omgeving een directe invloed zouden kunnen hebben op de darmbarrière, vooral op het niveau van genexpressie. We hebben bijvoorbeeld het effect van koolhydraten uit de voeding, darmbacteriën, fysiologische gassen en ontstekingsmediatoren op Caco-2-cellen getest (darmepitheelcellen) met behulp van het laboratoriummodel genaamd 'Human-oxygen Bacteria-anaerobic' of ook wel het 'HoxBan' systeem genoemd. Met het HoxBan-systeem waren we in staat om een kweek van ofwel een enkele bacteriesoort ofwel uit ontlasting afgeleide bacteriële groepen samen te kweken met menselijke darmepitheelcellen (Caco-2 cellen). Bovendien hebben we veel van onze bevindingen gevalideerd door de bacteriële en metabolietsamenstelling van menselijke ontlastingsmonsters te analyseren, evenals de genexpressieniveaus in darmbiopten van patiënten met IBD. Om de bevindingen van dit proefschrift gezamenlijk te ordenen, heb ik de wetenschappelijke inhoud in twee delen onderverdeeld: de darmmicrobiota en darmepitheelgezondheid (**Deel I**) en hypoxie, ontsteking en darmepitheelgezondheid (**Deel II**).

In het eerste deel van dit proefschrift heb ik me gericht op de communicatie en onderlinge afhankelijkheid tussen darmbacteriën en darmepitheelcellen van de mens. We beschrijven dat butyraat een belangrijke energiebron is voor het darmepitheel, maar er zijn zeker ook andere metabolieten die kunnen worden gebruikt voor epitheelgroei en -herstel, zoals enkelvoudige suikers (**Hoofdstuk 1**). We hebben zelfs aangetoond dat fermentatie van de onverteerbare vezel inuline door *F. prausnitzii* een overmaat aan fructose produceert, wat een koolstof- en energiebron is die gemakkelijk beschikbaar kan worden gesteld voor het menselijke dikke darmepitheel (**Hoofdstuk 2**). Bovendien presenteerden we de ontwikkeling van het laboratoriummodel HoxBan-Stool systeem,

dat de samen opkweken van levende uit ontlasting gehaalde groepen van bacteriën met darmepitheelcellen mogelijk maakte (**Hoofdstuk 3**). Hierin toonden we aan dat de toevoeging van inuline aan gevarieerde bacteriële samenstellingen de levensvatbaarheid en expressie van barrièrebeschermende genen in darmepitheelcellen verbeterde. De ontwikkeling van het laboratoriummodel HoxBan-Stool maakt het mogelijk om snel nieuwe geneesmiddelen te testen die mogelijk helpen bij het beïnvloeden en voorkomen van opvlammingen van ontstekingen bij patiënten met chronische darmontstekingsziekten.

Voor het tweede deel van dit proefschrift hebben we onze focus verlegd naar de micro-omgevingsregulatie van darmepitheelcellen in de context van IBD. In dit deel heb ik me specifiek gericht op een belangrijke factor die de darmwand (slijmvlies) omringt: een laag zuurstofgehalte of 'hypoxie'. Darmepitheelcellen vormen de barrière tussen de anaërobe (zuurstofarme) ruimte in de darm en het zuurstofrijke (geoxygeneerde) darmslijmvlies. De zuurstoftoevoer naar deze cellen is meestal minder dan wat ze eigenlijk nodig hebben, wat een fenomeen is dat bekend staat als fysiologische hypoxie. Dedarm epitheelcellen zijn heel dynamisch, want ze zijn zodanig geprogrammeerd dat ze zich kunnen aanpassen aan zulke lage zuurstofcondities, voornamelijk door de activering van een hypoxiegevoelig systeem dat wordt geregeld door de zogenaamde hypoxie-induceerbare factor 1-alfa (HIF1 α). In de situatie van hypoxie is de zuurstofbehoefte groter dan het zuurstofaanbod, waarbij de HIF1 α -route vervolgens wordt geactiveerd door de eiwitafbraak ervan te blokkeren. Dit brengt de genexpressie teweeg van tal van eiwitten die betrokken zijn bij onder meer metabolisme, vorming van bloedvaten (vascularisatie), de productie van extracellulaire matrixen de barrièrefunctie. Interessant genoeg leiden ontstekingsprocessen in het darmslijmvlies tot een nog hogere zuurstofbehoefte in verhouding tot het zuurstofaanbod, een proces dat bekend staat als pathofysiologische hypoxie.

In **Hoofdstuk 4** heb ik besproken wat er in de literatuur bekend is over factoren in het darmmilieu die de activering van de HIF1 α -route in darmepitheelcellen bepalen. Hierbij toonde ik aan dat hypoxie een belangrijk kenmerk is van het darmslijmvlies, dat zowel fysiologische als pathofysiologische processen in het darmslijmvlies sterk beïnvloedt. **Hoofdstuk 5** is gewijd aan de darm- -bacterie interactie en de implicaties ervan voor de activering van HIF1 α -afhankelijke genexpressie. We identificeerden het cytokine interleukine-18 als de belangrijkste boodschapper tussen de blootstelling van darmepitheelcellen aan de darmbacterie *F. prausnitzii* en de activering van de HIF1 α -route, wat beide potentiële factoren zijn om genezing van het darmslijmvlies bij IBD te kunnen bevorderen. In **Hoofdstuk 6** hebben we het effect van ijzertekort op HIF1 α -activering in het darmslijmvlies van patiënten met IBD bestudeerd, waarbij we hebben ontdekt dat de genexpressie van de zogenaamde transferrinereceptor (een ijzertransporter) afhankelijk is van de activering van de HIF1 α -route in de context van ontsteking en ijzertekort zoals voorkomt bij patiënten met IBD. Ten slotte is **Hoofdstuk**

7 gewijd aan het begrijpen van de invloed van ontsteking op de activering van de HIF1 α -route in het darmslijmvlies van patiënten met colitis ulcerosa. We hebben aangetoond dat de ontsteking van het darmslijmvlies via activering van de HIF1 α -route uiteindelijk leidt tot de totstandkoming van een barrière-beschermend effect. Alles bij elkaar genomen wijzen deze onderzoeken op de HIF1 α -route als een potentieel doelwit voor behandeling om de klinische symptomen en kenmerken van patiënten met IBD te verminderen.

Samenvattend laten de bevindingen van dit proefschrift zien dat de gezondheid van het menselijke darmslijmvlies direct wordt bepaald door de omgeving. We toonden aan dat darmbacteriën, voedselcomponenten, ijzertekort, ontsteking en hypoxie allemaal aspecten van de darmepitheelbarrière beïnvloeden die direct verband kunnen houden met de gezondheid van het darmslijmvlies. Het werk in dit proefschrift geeft inzicht in mogelijke therapeutische doelen die mensen kunnen helpen die lijden aan een ziekte waarbij de darmbarrière is verstoord, zoals bijvoorbeeld, maar zeker niet uitsluitend, IBD.

Resumo

O trato gastrointestinal (GI) ajuda a estabelecer a saúde de vários órgãos e sistemas em um organismo. O bem-estar humano pode estar diretamente associado à presença e abundância de diferentes espécies de microrganismos que habitam o trato GI, principalmente bactérias. Anteriormente conhecidas como flora, essas quase trinta trilhões de bactérias que colonizam cada um de nossos corpos são chamadas coletivamente de microbiota, e a maioria dessas bactérias está localizada no intestino grosso.

Células humanas precisam de oxigênio para sobreviver, porém, o lúmen intestinal torna-se anaeróbio no intestino grosso, local onde ocorre a maior parte da colonização de bactérias fermentadoras. A composição da microbiota intestinal é extremamente dinâmica e mudanças significativas são delimitadas pelo sexo, idade, hábitos alimentares e antecedentes genéticos de cada indivíduo. Algumas espécies bacterianas estão diretamente associadas à saúde humana, entre elas a bactéria anaeróbica *Faecalibacterium prausnitzii*. Por outro lado, o conteúdo que passa pelo nosso intestino (isto é, bolo alimentar) também pode conter componentes nocivos, como toxinas ou bactérias patogênicas. A fim de fornecer uma proteção física entre o bolo alimentar e nossa circulação sistêmica, as células epiteliais em nossos intestinos são altamente especializadas em criar uma firme barreira que permite a absorção seletiva de compostos do bolo alimentar, incluindo carboidratos, ácidos graxos, aminoácidos e proteínas. Açúcares simples, ácidos graxos e vitaminas são absorvidos no intestino delgado, enquanto, no intestino grosso, a fermentação bacteriana de componentes alimentares não-digeríveis gera nutrientes para o hospedeiro. De especial importância para a saúde humana, os ácidos graxos de cadeia curta (AGCC) são produzidos por fermentação bacteriana no intestino grosso. Existem vários tipos de AGCC, sendo o butirato o mais conhecido por desempenhar um papel fundamental na saúde humana, regulando a função da barreira intestinal, o metabolismo celular e exercendo efeitos anticancerígenos. *F. prausnitzii* é um exemplo de espécie bacteriana capaz de fermentar carboidratos complexos para produzir butirato, que é então absorvido por células epiteliais intestinais.

Mudanças na composição da microbiota intestinal estão ligadas a inúmeras doenças, incluindo diabetes tipo 2, câncer colorretal e a doença inflamatória intestinal (DII). DII é um termo genérico para descrever a doença de Crohn e a colite ulcerativa. DII é frequentemente diagnosticada pela primeira vez em uma idade jovem (abaixo dos 30 anos) e geralmente é seguida por períodos intermitentes de inflamação e remissão do trato GI. Essa inflamação ativa provoca dor abdominal, anemia, diarreia, constipação e pode eventualmente levar à ressecção cirúrgica do intestino do paciente. Infelizmente,

DII não pode ser curada no momento, mas grande atenção é dada ao efeito da microbiota na regulação da inflamação ou no prolongamento dos períodos de remissão. Pacientes com DII frequentemente apresentam baixos níveis de *F. prausnitzii* nas fezes, em comparação com indivíduos saudáveis. A grande questão que tentamos desvendar é como modular o meio ambiente intestinal para aliviar o processo inflamatório que esses pacientes sofrem.

Na minha tese, busquei desvendar fatores do ecossistema intestinal que impactam a função e a saúde de suas células epiteliais. Eu levantei a hipótese de que características específicas do microambiente intestinal saudável ou inflamado impactam diretamente a barreira intestinal, especialmente no nível de expressão gênica. Testamos o efeito de carboidratos dietéticos, bactérias intestinais, gases fisiológicos e mediadores inflamatórios em células epiteliais intestinais (Caco-2), usando o modelo *in vitro* Human-oxygen Bacteria-anaerobe (ou HoxBan). Com o sistema HoxBan, fomos capazes de co-cultivar culturas puras de uma única espécie bacteriana ou comunidades bacterianas derivadas de fezes com células intestinais humanas (Caco-2). Além disso, validamos muitas de nossas descobertas analisando a composição bacteriana e metabólica de amostras de fezes humanas, bem como os níveis de expressão gênica de biópsias intestinais de pacientes com DII. Para organizar coletivamente os achados desta tese, dividi o conteúdo científico em duas partes: microbiota intestinal e saúde do epitélio intestinal (**Parte I**) e hipóxia, inflamação e saúde do epitélio intestinal (**Parte II**).

Na primeira parte desta tese, concentrei-me na comunicação e interdependência entre os micróbios intestinais e as células epiteliais intestinais do hospedeiro. Descrevemos que o butirato é uma importante fonte de energia para o epitélio, mas certamente existem outros metabólitos que podem ser usados para o crescimento e reparo epitelial, como os açúcares simples (**Capítulo 1**). De fato, mostramos que a fermentação da fibra não-digerível inulina por *F. prausnitzii* produz excesso de frutose, uma fonte de carbono e energia que está prontamente disponível para o epitélio intestinal humano (**Capítulo 2**). Além disso, apresentamos o desenvolvimento do sistema *in vitro* HoxBan-Stool, que permitiu o co-cultivo de comunidades microbianas derivadas de fezes com células epiteliais intestinais (**Capítulo 3**). Aqui, mostramos que a suplementação de inulina para comunidades bacterianas aumentou a viabilidade e a expressão de genes envolvidos na formação da barreira por células epiteliais intestinais. O desenvolvimento do modelo *in vitro* HoxBan-Stool permite testes rápidos de novos medicamentos que potencialmente ajudarão a modular e prevenir crises inflamatórias em pacientes com doenças inflamatórias do trato GI.

Para a segunda parte desta tese, mudamos nosso foco para a regulação microambiental das células epiteliais intestinais no contexto da DII. Nesta parte, concentrei-me especificamente em uma característica importante da parede (mucosa) intestinal: baixo oxigênio ou “hipóxia”. As células epiteliais intestinais formam a barreira entre o lúmen do intestino anaeróbico e a mucosa intestinal oxigenada, de modo

que a oferta de oxigênio a essas células costuma ser menor do que sua demanda, em um fenômeno conhecido como hipóxia fisiológica. Por formarem uma monocamada altamente dinâmica, as células epiteliais intestinais são programadas para se adaptar a essas condições de baixo oxigênio, principalmente devido à ativação da via sensível à hipóxia que é controlada pelo fator induzível por hipóxia-1 alfa (HIF1 α). Em hipóxia, a demanda de oxigênio excede a oferta, ativando a via HIF1 α ao bloquear sua degradação proteica, o que desencadeia a expressão gênica de inúmeras proteínas envolvidas no metabolismo, vascularização, produção de matriz extracelular e função de barreira, entre outros. Curiosamente, processos inflamatórios na mucosa intestinal causam maior demanda e menor oferta de oxigênio ao tecido, em um processo conhecido como hipóxia fisiopatológica.

No **Capítulo 4**, revisei o que é conhecido na literatura sobre fatores no ecossistema intestinal que determinam a ativação da via HIF1 α nas células epiteliais intestinais. Mostrei que a hipóxia é uma característica chave da mucosa intestinal, que influencia fortemente os processos fisiológicos e fisiopatológicos. O **Capítulo 5** é dedicado à interação intestinal entre hospedeiro-micróbio e sua implicação na ativação da expressão gênica dependente de HIF1 α . Identificamos a citocina interleucina-18 como o principal mensageiro entre a exposição das células epiteliais intestinais à bactéria intestinal *F. prausnitzii* e a ativação da via HIF1 α , que são alvos potenciais para promover a cicatrização da mucosa em pacientes com DII. No **Capítulo 6**, estudamos o efeito da deficiência de ferro na ativação da HIF1 α na mucosa intestinal de pacientes com DII, identificando assim a expressão gênica do transportador de ferro receptor 1 da transferrina como dependente da ativação da via HIF1 α no contexto da inflamação e deficiência de ferro em DII. Por fim, o **Capítulo 7** é dedicado a compreender o impacto da inflamação na ativação da via HIF1 α na mucosa intestinal em pacientes com colite ulcerativa. Caracterizamos que a inflamação da mucosa intestinal desencadeia a ativação da via HIF1 α para um efeito protetor da barreira intestinal. Em conjunto, esses estudos apontam para a via HIF1 α como um alvo potencial para tratar as manifestações da DII em pacientes.

Em resumo, os achados desta tese demonstram que a saúde da mucosa intestinal humana é determinada diretamente pelo seu micro ambiente. Mostramos que micróbios intestinais, alimentos, deficiência de ferro, inflamação e hipóxia influenciam aspectos da barreira epitelial que podem estar diretamente ligados à saúde da mucosa intestinal. O trabalho nesta tese lança luz sobre alvos terapêuticos que podem ajudar pacientes com doenças da barreira intestinal, como, mas não exclusivamente, DII.

INTRODUCTION

1. Rationale

An intact yet dynamic epithelial barrier of the intestine is central to gut and systemic health. The intestinal barrier is a monolayer of heterogeneous and highly specialized cells that provide a physical obstacle between the luminal content and our systemic circulation, tissues and organs. Disruption of the intestinal barrier is often observed in inflammatory diseases of the gastrointestinal (GI) tract, such as inflammatory bowel disease (IBD). It is therefore essential to know what controls the integrity of this dynamic barrier, but there is still limited understanding of the factors that regulate intestinal barrier homeostasis. The microenvironment of the GI tract is complex and directly impacts health and disease. Gut microbiota, physiologic gases and mediators of inflammatory response are key players in regulating intestinal epithelial health. Understanding these microenvironmental factors will improve our knowledge and generate potential targets for drug development.

2. What is the intestinal microenvironment?

The intestinal microenvironment harbors a collection of factors to which intestinal mucosal cells (epithelial and resident immune cells) are exposed to. These factors may come from the gut lumen, systemic blood circulation, neighboring cells and disease conditions (**Figure 1**). In this thesis, we will focus on 3 microenvironmental factors that directly affect intestinal epithelial health: the gut microbiota, oxygen availability and inflammation.

2.1. Microbiota

The large intestine harbors a wide array of microorganisms that are collectively named gut microbiota. The bacteria in the gut microbiota are responsible for the fermentation of undigestible food components, which in turn generates metabolites that are partly absorbed by the cells in the colonic lining (mostly epithelial cells) and act as energy source, immune modulators and signaling molecules to a variety of organs and tissues. Significant changes in the composition of the gut microbiota (i.e., dysbiosis) are associated to the pathophysiology of a number of diseases, especially IBD [1]. However, it remains a challenge to determine the cause-consequence relationship between, for instance, dysbiosis and pathophysiological process, such as (intestinal) inflammation.

The lumen of the large intestine is a largely anaerobic environment where the fermentation of undigestible food components is performed by (strict and facultative) an-

Figure 1. Microenvironmental determinants of intestinal epithelial homeostasis. The colonic mucosa is in close contact with the anoxic, microbial-populated, gut lumen, creating an oxygen gradient starting from the highly perfused subepithelial mucosa. Molecules and minerals (of special importance, iron (Fe)) are exchanged from the systemic circulation to the mucosa by the capillaries. Metabolites from bacterial digestion of fibers, such as SCFAs and sugars are by absorbed and transported across the epithelial barrier of the intestine. Resident immune cells (as well as epithelial) regulate mucosal homeostasis via the production and secretion of numerous cytokines.

aerobic bacteria [2]. The gut microbiota is mostly composed of species from five dominant phyla: Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia, of which Bacteroidetes and Firmicutes combined comprise around 90% of the gut microbiota in a healthy adult individual [3]. The firmicute *Faecalibacterium prausnitzii* is one of the most dominant single bacterial species in the healthy gut and has been widely associated to human health, with its abundance generally reduced in patients with IBD [4,5]. *F. prausnitzii* helps in the digestion of complex polymeric carbohydrates (fibers), among other, producing butyrate as end-product. Butyrate is a short-chain fatty acid (SCFA) that has been associated to epithelial health by improving epithelial barrier function and functioning as signaling molecule and energy source for colonic intestinal epithelial cells (colonocytes) [6–9].

2.2. Oxygen availability

The epithelial cells that line the intestinal mucosa are in a constant state of “physiological” hypoxia, since these cells are positioned at the interphase between the anaerobic gut lumen and the highly perfused mucosa [10]. Hypoxic conditions activate the transcriptional factors hypoxia-inducible factor 1-alpha (HIF1 α) and 2-alpha (HIF2 α), an iron- and oxygen-sensitive pathway that regulates the expression of numerous genes involved in metabolism, barrier formation and immune signaling processes. HIF1 α is constitutively expressed and its transcriptional function is regulated by a group of asparaginyl- and prolyl-hydroxylases (FIH and PHD1-3, respectively).

When cellular oxygen supply exceeds demand, FIH and PHD enzymes utilize oxygen as a cofactor for the hydroxylation of HIF1 α , which is then recognized by the von-Hippel-Lindau protein (VHL) leading to proteasomal degradation of HIF1 α . In contrast, when cellular oxygen demand exceeds supply, oxygen is not sufficiently available for the hydroxylase reaction by FIH or PHD. Consequently, HIF1 α protein stabilizes and translocates to the nucleus, where it drives the transcription of a number of genes, by binding to hypoxia-responsive elements (HREs) in their promoter regions. Interestingly, butyrate was previously shown to induced HIF1 α activation in intestinal epithelial cells, increasing the expression of barrier function proteins, which stimulates regeneration of the intestinal lining [11].

2.3. Inflammation

The gut lining is constantly exposed to exogenous substances derived from diet and the gut microbiota. Some argue that this generates a continuous low-grade inflammatory status in the intestinal epithelial cells. Numerous cytokines are secreted by the intestinal mucosa, modulating proliferation, apoptosis and barrier function of intestinal epithelial cells [12]. Nonetheless, this delicate homeostatic balance that maintains the correct function of the intestinal epithelium can be disturbed, for instance by inflammatory conditions in the intestinal mucosa as observed in patients with IBD. IBD is an umbrella term that encompasses Crohn's disease and ulcerative colitis, both diseases characterized by recurring and life-lasting inflammatory flares in the GI tract, especially in the small and large intestines [13]. Furthermore, patients with IBD often show extra-intestinal manifestations of the disease, especially iron deficiency anemia. The molecular implications of low systemic iron levels, in particular relating to possible effects on the HIF pathways, still represents a gap in our knowledge [14,15]. Interestingly, inflammation drives a more extensive and severe state of pathophysiological hypoxia and activation of the HIF1 α pathway has regenerative effects on the gut lining in several *in vivo* animal models of colitis [16]. The work presented in **Chapter 7** demonstrates for the first time in IBD patients the interrelationship between inflammation and hypoxia, showing that the inflamed mucosa of patients with ulcerative colitis become severely hypoxic, triggering transcriptional responses towards protective HIF1 α activation in the intestinal mucosa. My contribution to that publication founded my interest in the role of HIF signaling in the complex environment of the human gut and forms the basis of much that is presented in this thesis.

3. Why is it important to regulate intestinal epithelial health?

The goal of studying the microenvironmental regulation of epithelial health is to characterize the molecular mechanism involved and to define targets that can be modulated to improve gut health. Increasing the molecular medical knowledge of the cellular host-microenvironmental interaction in the intestine provides new insights and targets to prevent flares and improve the quality of life of patients with gut-related diseases, such as IBD. For example, Western diet (rich in sugars, fat, animal protein and

processed food) is recognized as trigger that leads to dysbiosis, which, in turn, is associated with the pathophysiology of IBD [17]. However, it still remains a challenge to provide a causal link between microenvironmental changes and intestinal epithelial health. Defining such cause-consequence relationships in experimental systems is difficult, mainly due to the complexity of the intestinal anatomy with its oxic-anoxic interphase and the rich anaerobic bacteria composition in the gut microbiota. In this thesis, our goal is to study the functional interrelationship between intestinal microenvironmental factors (e.g., gut bacteria, systemic iron levels, inflammation and hypoxia) and intestinal epithelial health.

Accumulating evidence is pointing to a central role of oxygen-sensing in the inflammatory response, as stabilization of HIF1 α protein has protective outcomes in different inflammatory diseases [18]. An oral hydroxylase inhibitor (Roxadustat; FG-4592) showed clinical benefits in a randomized Phase 3 clinical trial for treating anemia of chronic kidney disease [19–21], due to the erythropoietic effects of HIF1 α . The application of Roxadustat for anemia of chronic kidney failure has recently been authorized to use in the European Union [22], which might also represent a stepping stone on the management of IBD.

4. Aims & Outline

The governing hypothesis of this thesis is that the gut microenvironment determines intestinal epithelial and mucosal homeostasis. In order to test this hypothesis, we designed three specific aims:

AIM 1: The role of microbial metabolism in intestinal epithelial homeostasis

In this section, we aimed to test the impact of microbial metabolism on intestinal epithelial health. In **Chapter 1**, we aimed to present an overview of the relevant literature regarding bacteria-to-epithelium cross-feeding in the intestine, especially regarding the bacterial metabolites used by intestinal epithelial cells as energy and/or carbon sources. **Chapter 2** aimed to characterize *F. prausnitzii*-to-intestinal epithelium cross-feeding at the molecular level, mimicking the colonic fiber-rich environment. In **Chapter 3**, our goal was to develop an *in vitro* model (the HoxBan-Stool system) that allows the coculture of human intestinal epithelial cells with complex gut microbiota-derived bacterial communities.

AIM 2: The role of oxygen sensing on intestinal epithelial homeostasis

In this section, we aimed to test the impact of oxygen sensing, specifically the HIF1 α activation, on intestinal epithelial health. In **Chapter 4**, we provide a literature review that describes the microenvironmental factors in the intestine that modulate mucosal HIF1 α activity, with special focus on barrier function and epithelial regeneration. In **Chapter 5**, we aimed to investigate the correlation between gene expression of the epithelial-derived cytokine IL-18 and bacterial-driven activation of HIF1 α pathway in the intestinal mucosa and epithelium of patients with IBD.

AIM 3: The role of inflammation on intestinal mucosal homeostasis

In this section, we aimed to test the impact of inflammation on intestinal mucosal health, especially related to epithelial HIF1 α activation. In **Chapter 6**, our goal was to analyze the correlation between the gene expression of intestinal regulators of iron metabolism (such as the major iron transporter *TFR*C), systemic iron deficiency and intestinal mucosal inflammation in patients with IBD, using *in vitro*, clinical and transcriptional data from 101 patients with IBD. In **Chapter 7**, we aimed to analyze the impact of inflammation on intestinal mucosal HIF1 α (and 2 α) activation capacity in a cohort of patients with ulcerative colitis.

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Part I

Gut microbiota and intestinal epithelial health