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Glicogenoses hepáticas: o microbioma intestinal como fator de influência

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Lista de Abreviaturas

AGCC: Ácido graxo de cadeia curta
AMC: Amido de milho cru
BMP: *Brazilian Microbiome Project*
CEP: Comitê de Ética em Pesquisa
EIM: Erros inatos do metabolismo
G6P: Glicose-6-fosfato
G6Pase: Glicose-6-fosfatase
G-CSF: *Granulocyte Colony-Stimulating Factor*
GM-CSF: *Granulocyte/Macrophage Colony Stimulating Factor*
GPCR: receptores acoplados à proteína-G (*G-Protein-Coupled Receptors*)
GPPG: Grupo de Pesquisa e Pós-Graduação
GRO: *Growth-Regulated Oncogene*
GSD: Doenças do armazenamento do glicogênio (*Glicogen Storage Diseases*)
HCA: Adenoma hepatocelular (*hepatocellular adenoma*)
HCC: Carcinoma hepatocelular (*hepatocellular carcinoma*)
HCPA: Hospital de Clínicas de Porto Alegre
IBD: doença inflamatória intestinal (*Inflammatory Bowel Disease*)
IEM: *Inborn Errors of Metabolism*
IFN-γ: Interferon-γ (*Interferon-γ*)
IgA: Imunoglobulina A (*Immunoglobulin A*)
IL-10: Interleucina-10 (*Interleukin-10*)
IL-13: Interleucina-13 (*Interleukin-13)*
IL-17A: Interleucina-17A (*Interleukin-17A*)
IL-1α: Interleucina-1α (*Interleukin-1α*)
IL-1β: Interleucina-1β (*Interleukin-1β*)
IL-4: Interleucina-4 (*Interleukin-4*)
IL-6: Interleucina-6 (*Interleukin-6*)
IL-8: Interleucina-8 (*Interleukin-8*)
IP-10/CXCL10: *Interferon gamma-induced protein-10/C-X-C motif chemokine 10*
IPP: Interação proteína-proteína

LPS: lipopolissacarídeo (*Lipopolysaccharide*)

MCP-1/CCL2: *Monocyte Chemoattractant Protein-1/C-C Motif Chemokine Ligand 2*

MDC/CCL22: *Macrophage-Derived Chemokine/C-C Motif Chemokine Ligand 22*

MIP-1α/CCL3: *Macrophage Inflammatory Protein-1α/C-C Motif Chemokine Ligand 3*

MIP-1β/CCL4: *Macrophage Inflammatory Protein-1β/C-C Motif Chemokine Ligand 4*

OTU: Unidades taxonômicas operacionais (*Operational Taxonomic Unit*)

PCR: Reação em cadeia da polimerase (*Polymerase Chain Reaction*)

pH: Potencial hidrogeniônico

PPI: Interação proteína-proteína (*Protein-Protein Interaction*)

SCFA: Ácido graxo de cadeia curta (*Short-Chain Fatty Acid*)

SGM: Serviço de Genética Médica

SSIEM: *Society for the Study of Inborn Errors of Metabolism*

TCLE: Termo de Consentimento Livre e Esclarecido

TLR4: Receptor do tipo Toll-4 (*Toll-Like Receptor 4*)

TNF-α: Fator de necrose tumoral-α (*Tumor Necrosis Factor-α*)

TNF-β: Fator de necrose tumoral-β (*Tumor Necrosis Factor-β*)

TRI: Triglicerídeos

UBS: Unidade Básica de Saúde

VEGF: Fator de crescimento endotelial vascular (*Vascular Endothelial Growth Factor*)

UPR^{mt}: resposta mitocondrial a proteínas mal-dobradas (*Mitochondrial Unfolded Protein Response*)

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1 RESUMO

Introdução: O microbioma é o conjunto de microrganismos, metabólitos, genes e ambiente no qual estão inseridos. O microbioma interage com o hospedeiro através de processos metabólicos e imunes, sendo influenciado pela dieta, genética, idade, níveis hormonais, medicamentos e geografia. O desequilíbrio da microbiota intestinal (disbiose) está associado a doenças frequentes nas glicogenoses hepáticas (GSD), tais como obesidade, doença inflamatória intestinal (IBD), diabetes, osteoporose e esteato-hepatite não alcóolica. As GSD, uma classe de erros inatos do metabolismo (EIM), são doenças genéticas raras do metabolismo do glicogênio. Esta tese foca nas GSD-Ia (*G6PC*), GSD-Ib (*SLC37A4*), GSD-III (*AGL*) e GSD-IX (*PHAK*), as quais são caracterizadas por defeitos na rota de degradação e são majoritariamente tratadas por intervenção dietética, com restrição de carboidratos de hidrólise rápida e administração frequente e periódica de amido de milho cru (AMC).

Objetivo: Caracterizar a relação entre microbioma intestinal e erros inatos do metabolismo, com ênfase nas glicogenoses hepáticas.

Metodologia: O estudo foi desenvolvido em quatro etapas. Etapa 1: revisão não sistemática da literatura tanto sobre microbiota intestinal e EIM, quanto microbiota e as classes de tratamentos mais utilizados nos EIM. Etapa 2: estudo experimental, transversal, controlado, com amostragem de conveniência, incluindo 24 pacientes geneticamente diagnosticados com GSD em tratamento com AMC e 16 controles hígidos. Os participantes deveriam ter idade ≥ 3 anos, sem sinais de infecção, não ter sido vacinado nos 15 dias antecedentes às coletas ou estar em tratamento com antibióticos, não ter sido indicado ou recebido transplante de órgãos. Controles deveriam ter mesmo sexo e idade similar (±1 ano) aos pacientes. Amostras fecais e de sangue foram coletadas, e adicionalmente os participantes preencheram um registro alimentar de três dias e responderam a um questionário clínico. O DNA bacteriano fecal foi extraído e realizou-se o sequenciamento parcial do gene *16S rRNA* na plataforma Ion Torrent. Os dados do sequenciamento foram curados segundo a metodologia do BMP, classificados taxonomicamente, analisados em ambiente R (pacotes vegan

e phyloseq) e com o software MicrobiomeAnalyst. Os nutrientes da dieta foram quantificados com o software Nutribase e o pH fecal foi aferido com pHmetro eletrônico. A calprotectina fecal foi quantificada com kit comercial do tipo ELISA segundo as instruções do fabricante. Etapa 3: estudo experimental, transversal, controlado, com amostragem de conveniência, incluindo 27 pacientes (GSD-Ia=16, GSD-Ib=06, GSD-III=02, GSD-IX=03) e 24 controles. Os critérios de inclusão foram os mesmos utilizados na etapa 2. Após a coleta de sangue dos participantes, 20 citocinas plasmáticas (G-CSF, GM-CSF, IFNy, GRO, IL-10, MDC, IL-13, IL-17A, IL-1α, IL-1β, IL-4, IL-6, IL-8, IP-10, MCP-1, MIP1α, MIP-1β, TNFα, TNFβ e VEGF) foram quantificadas com kit comercial Multiplex Cytokine Assay (Luminex). Pacientes GSD-I, III e IX foram comparados aos controles, e os tipos de GSD foram comparados entre si, considerando características clínicas. Etapa 4: Análise *in silico* através de redes de IPP, utilizando-se os identificadores SLC37A4, G6PC, G6PC2, G6PC3, ITGAL, ITGAM, S100A8 e S100A9 como dados de entrada na ferramenta STRING. O software CytoScape foi utilizado para a análise de módulos, medidas de centralidade, ontologias e para a construção de redes secundárias de interação.

Resultados: Etapa 1: Um dos principais fatores de associação entre microbioma intestinal e EIM é a dieta/tratamento, que normalmente é restrita e/ou sobrecarregada de forma específica nos EIM. Além disso, o microbioma intestinal possui forte associação com o fígado e cérebro, dois órgãos mais afetados nos EIM. Etapa 2: pacientes possuem uma menor diversidade e estrutura do microbioma fecal diferente dos controles em vários níveis taxonômicos. Pacientes apresentaram prevalência maior ($p \leq 0,05$) de IBD, obesidade/sobrepeso, consumo de medicamentos e calorias (majoritariamente do AMC) do que o grupo controle. Além disso, pacientes consumiam menos gorduras, cálcio, sódio e vitaminas, e apresentaram pH fecal mais ácido. A riqueza microbiana teve correlação com o pH ($r=0,77$; $p=6,8e-09$), consumo de carboidratos totais ($r=0,6$; $p=4,8e-05$) e açúcar ($r=0,057$; $p=0,00013$). Etapa 3: pacientes apresentaram níveis reduzidos ($p \leq 0,05$) de IL-4, MIP-1α, MDC, TNF-β e VEGF em comparação aos controles. Entre os pacientes, os níveis de citocinas diferiram ($p \leq 0,05$) conforme o tipo de GSD, presença de anemia, adenoma e níveis de triglicerídeos. Etapa 4: Nas redes de interação exclusivas da GSD-Ia, várias proteínas se conectaram aos nós

centrais. Dentre as proteínas, selecionou-se como de interesse TLR4, ITGAL, ITGAM, IL-6 e IL-10, os quais podem exercer um importante papel na interação entre microbiota, sistema imune e saúde do hospedeiro dentro do contexto das GSD.

Conclusões: Pacientes com GSD hepática apresentam disbiose intestinal e um desequilíbrio na imunomodulação. Através das redes IPP foi possível identificar que as interações proteicas provavelmente alteradas na GSD-Ia compreendem proteínas relacionadas ao sistema imune, as quais podem atuar como moduladores na interação entre microbioma e hospedeiro.

1 ABSTRACT

Introduction: The microbiome is defined as the entire habitat, including the microorganisms, their genomes and the surrounding environmental conditions. The gut microbiome interacts with the host mainly through metabolic and immunological processes and can be affected by several factors, such as diet, host genetics, age, sex, medicines, and geographic factors. Dysbiosis, an imbalance in the gut microbiome, is related to several multifactorial diseases like obesity, inflammatory bowel disease (IBD), diabetes, osteoporosis and non-alcoholic fatty liver disease, which is commonly developed by patients with hepatic glycogen storage diseases (GSD). GSD are rare genetic diseases, a class of inborn errors of metabolism (IEM) that affect glycogen metabolic pathways. This study focuses on GSD-Ia (*G6PC*), GSD-Ib (*SLC37A4*), GSD-III (*AGL*) and GSD-IX (*PHKA*), all of them with an impaired degradation pathway. These GSD are mainly treated by dietetic intervention, restriction of fast-hydrolysis carbohydrates and frequent and periodic ingestion of uncooked cornstarch (UCCS).

Objectives: To characterize the relation between the intestinal microbiota and IEM, with emphasis on hepatic GSD.

Methods: This study was developed in four phases. Phase 1: Non-systematic review of studies about the gut microbiota and IEM, as well as the gut microbiota and the most common treatments for IEM. Phase 2: Experimental, cross-sectional, controlled study with 24 patients genetically diagnosed for hepatic GSD on UCCS treatment and 16 healthy controls sampled by convenience. As inclusion criteria, subjects should be 3 years or older, not presenting signs of infection, not vaccinated 15 days prior to samples collection, not be on antibiotics, not have been designated to receive or received organ transplant. Controls should be of similar sex and age (±1 year) to the patients. Fecal and blood samples were collected and subjects filled a three-day food record and answered a clinical questionnaire. After fecal DNA extraction and partial 16S rRNA sequencing, results were analyzed according to the BMP recommendations, taxonomically classified, analyzed on R environment (phyloseq and vegan packages) and Microbiome Analyst software. The nutrient intake was estimated through the Nutribase software, and the pH was measured by an electronic pH-meter. Fecal

calprotectin quantification was performed through commercial ELISA kit. Phase 3: Experimental, cross-sectional, controlled study with 27 patients with genetic diagnosis for hepatic GSD on UCCS treatment (Ia=16, Ib=6, III=2, IX=3) and 24 healthy controls sampled by convenience. The inclusion criteria were the same as used in phase 2. Subjects collected 5 mL of blood and 20 blood cytokines (G-CSF, GM-CSF, IFNy, GRO, IL-10, MDC, IL-13, IL-17A, IL-1α, IL-1β, IL-4, IL-6, IL-8, IP-10, MCP-1, MIP1α, MIP-1β, TNFα, TNFβ e VEGF) were quantified through a multiplex assay kit. Patients (I, III and IX) were compared with controls, and the GSD types were compared taking into account clinical features. Phase 4: *In silico* analysis of PPI networks using the identifiers/interactors SLC37A4, G6PC, G6PC2, G6PC3, ITGAL, ITGAM, S100A8 and S100A9 as input on STRING. CytoScape software was used to analyze modular structure, centrality measures and gene ontology terms.

Results: Phase 1: The main factor of association between IEM and the gut microbiome is the diet/treatment, which is restricted/overloaded in a specific way on IEM. The gut microbiota has strong association with the liver and the brain, two of the most affected organs on IEM. Phase 2: The GSD microbiome was characterized by low diversity and distinct microbial structure. Patients had higher prevalence of IBD and overweight/obesity, usage of medicines, higher calories' intake, mainly due the UCCS, and lower intake of fat, calcium, sodium, and vitamins than controls. Patients presented lower fecal pH than controls. The OUT abundance was correlated with fecal pH ($r=0.77$; $p=6.8e-09$), total carbohydrate ($r=-0.6$; $p=4.8e-05$) and sugar ($r=0.057$; $p=0.00013$) intakes. Phase 3: Patients presented reduced ($p\leq0.05$) levels of IL-4, MIP-1α, MDC, TNF-β and VEGF. Cytokine level were different among patients according to GSD type ($p\leq0.05$), presence of anemia, adenoma and triglyceride levels. Phase 4: The merged PPI network corresponding GSD-Ia exclusive network presented several interactions among immune system components. As interesting interactors were identified TLR4, ITGAL, ITGAM, IL-6 and IL-10.

Conclusion: The GSD patients presented dysbiosis and an imbalance on immunomodulation. Through PPI networks it was possible to identify that altered PPI in GSD-Ia involving several proteins related to innate immune system, which can act as modulators on host-microbiome interaction.

2 INTRODUÇÃO

2.1 O microbioma humano

Nós nunca fomos indivíduos (Gilbert et al. 2012). A ideia de que humanos são seres holobiontes, em uma relação simbiótica com diversos microorganismos, vem ganhando força nos últimos anos. Não só as ciências naturais, mas também as ciências sociais- inclusive a filosofia e a sua incansável procura sobre a essência do ser- ganharam novos ares com o estudo do microbioma (Rees et al. 2018).

O microbioma considera fatores ambientais bióticos e abióticos, e pode ser definido como o habitat inteiro, incluindo microrganismos (Archaea, bactérias, fungos e vírus), seus genomas e as condições ambientais que o cercam. O microbioma é caracterizado pela aplicação de uma ou mais "-ômicas" (metagenômica, metabolômica, metatranscriptômica, metaproteômica) combinadas com dados clínicos e/ou ambientais (Marchesi and Ravel 2015).

Em uma revisão atual, estima-se que o número de bactérias no corpo humano seja em torno de $3,8x10^{13-14}$, de mesma ordem que o número de células humanas, contendo uma massa aproximada de 0,2 kg (tendo como referência um homem de 70 kg) (Sender et al. 2016), e abrigando de 500-1.000 espécies bacterianas simultaneamente, as quais codificam em torno de 2.000 genes cada, superando, no total, em 100x os cerca de 20.000 genes codificados pelo organismo humano (Turnbaugh et al. 2007).

O número de bactérias e os próprios fatores abióticos, tais como pH, possuem uma grande variação no corpo humano. Assim sendo, existe variedade intraindividual (Grice et al. 2009; Human Microbiome Project Consortium 2012), conforme a parte do corpo em que o microbioma está inserido e também interindivíduos, possuindo caráter próprio para microbiomas isolados de uma mesma parte do corpo de diferentes pessoas (Franzosa et al. 2015).

Até recentemente, assumia-se que o ambiente fetal era estéril, e que a colonização tinha início ao nascimento, com a passagem do bebê pelo canal vaginal materno. Entretanto, com o surgimento de tecnologias que permitiram a identificação de microrganismos sem a necessidade de cultura, após a detecção de microrganismos no cordão umbilical (Jiménez et al. 2005), placenta (Aagaard et al. 2014), líquido amniótico, membranas fetais (Steel et al. 2005) e mecônio (Stinson et al. 2019), levantou-se a possibilidade de que a colonização microbiana pode ser iniciada ainda no período intrauterino por transferência materna, embora alguns dados ainda sejam controversos (Theis et al. 2019) .

Durante os três primeiros anos de vida o microbioma muda drasticamente, a partir de quando atinge a maturidade e tende a permanecer relativamente estável durante a idade adulta (Koenig et al. 2011), sendo únicos para cada pessoa. Embora únicos, ao longo da vida existirão flutuações nas taxas de crescimento e sobrevivência dos microrganismos em resposta a perturbações (mudança de dieta, uso de medicações, infecções, estilo de vida, *etc*), mas a microbiota e a estrutura original são resilientes, e tenderão a retornar a um estado similar ao anterior à perturbação, uma vez que cesse o estímulo (David et al. 2014a). Existe, portanto, uma estabilidade em curto e médio prazo, na ausência de agentes estressores (Voigt et al. 2015). Entretanto, em longo prazo, o microbioma sofre mudanças, variando conforme a idade (Odamaki et al. 2016; Yatsunenko et al. 2012). Dentre os fatores que influenciam o microbioma, a genética e a dieta são de extrema importância e serão discutidos de forma mais detalhada nas seções posteriores.

2.1.1 O microbioma intestinal humano

O cólon abriga a maior parte do conteúdo bacteriano humano, contendo aproximadamente 10^{14} bactérias (Sender et al. 2016). As bactérias intestinais possuem funções metabólicas importantes, e fazem parte do metabolismo de glicanos, aminoácidos e xenobióticos, produção de metano, biossíntese de vitaminas (via metabólica 2C-metil-D-eritritol-4-fosfato) e isoprenóides (Gill et al. 2006). O metabolismo que entendemos como humano, na verdade compreende

um conjunto das atividades metabólicas humanas e bacterianas (Bäckhed et al. 2005).

A microbiota intestinal exerce funções de proteção, manutenção estrutural e metabólicas, e interfere diretamente na saúde do hospedeiro (O’Hara and Shanahan 2006). Estas interações serão abordadas de forma mais detalhada no tópico 2.2. A microbiota intestinal de um adulto é composta tipicamente de poucos filos, nos quais *Bacteroidetes* e *Firmicutes* são dominantes, seguidos por *Proteobacteria*, *Verrucomicrobiota*, *Actinobacteria* e *Euryarchaeota* (Eckburg et al. 2005). Destes filos, *Bacteroidetes* e *Actinobacteria* tendem a ser mais estáveis, enquanto *Firmicutes* e *Proteobacteria* são mais suscetíveis a perturbações. A presença de determinados filos é consistente em longo prazo, embora suas abundâncias sejam sujeitas a mudanças (Faith et al. 2013). Entender como a microbiota intestinal afeta os estados de saúde e doença requer uma abordagem ecológica, considerando a comunidade como um todo e, portanto, um certo abandono do foco em patógenos individuais (Lozupone et al. 2012).

2.1.1.1 A dieta e o microbioma intestinal

A dieta e o microbioma estão intimamente relacionados, e a dieta o principal modulador do microbioma intestinal. A influência da dieta sobre o microbioma tem início logo após o nascimento, com diferenças consideráveis entre os bebês alimentados exclusivamente com leite materno e aqueles alimentados de forma não-exclusiva. A diversidade, processos de maturação e abundância relativa de *Bacteroidetes* e *Firmicutes* são mais altos naquelas crianças alimentadas de forma não exclusiva, enquanto vias relacionadas ao metabolismo de vitaminas, lipídios e detoxificação são diminuídas neste mesmo grupo (Ho et al. 2018). De acordo com a meta-análise realizada por Ho e colaboradores (2018), um longo período de amamentação exclusiva também está associado com uma diminuição dos episódios de diarreia relacionados à disbiose intestinal, e essas diferenças não são restritas aos seis primeiros meses, permanecendo pelo menos até os dois anos de idade. Há, em bebês alimentados de forma exclusiva com leite materno por menos de dois meses, uma menor abundância relativa de

Actinobacteria, e o aumento de *Firmicutes*, bem como a queda na abundância relativa de *Bifidobacteriaceae* e *Enterococcaceae* e o aumento de *Lactobacillaceae*, *Coriobacteriaceae*, *Prevotellaceae*, *Clostridiaceae*, *Erysipelotrichaceae* e *Lachnospiraceae* (Ho et al. 2018).

Além do tempo de amamentação exclusiva, o uso de fórmulas interfere na estrutura e diversidade do microbioma intestinal (Bezirtzoglou et al. 2011; Lee et al. 2015; Azad et al. 2016), e pode contribuir para o sobrepeso na infância (Forbes et al. 2018). Bebês alimentados exclusivamente com leite materno até os seis meses de idade possuem uma maior resiliência do microbioma intestinal e sucessão ecológica contínua, sendo menos afetados por fatores externos, tais como alimentação complementar e antibióticos (Carvalho-Ramos et al. 2018).

A composição do microbioma muda drasticamente nos primeiros anos de vida. De maneira geral, dos 0 aos 9 meses de idade, bebês alimentados no peito possuem como maiores componentes da microbiota *Actinobacteria* e *Firmicutes*, enquanto em bebês alimentados por fórmulas predominam Actinobacteria e *Bacteroidetes*. Entretanto, independentemente de ser amamentado no peito ou por fórmulas, durante este período ainda existem poucas espécies bacterianas habitando o intestino. Dos 9 aos 18 meses, há o primeiro grande aumento de diversidade, com um aumento de bactérias produtoras de butirato e com dominância de *Bacteroidetes* e *Firmicutes*. Neste período ainda existe instabilidade devido ao grande fluxo da composição bacteriana. Já dos 18 aos 36 meses ocorre a estabilização da formação do microbioma intestinal, com um novo aumento da diversidade das espécies bacterianas e da produção de butirato, com predominância de *Bacteroidetes* e *Firmicutes*, embora haja uma variação grande na abundância de *Prevotella* e *Firmicutes* dependendo do tipo de dieta adotada (Voreades et al. 2014).

Os seis meses de idade são um ponto-chave na maturação da microbiota intestinal pois normalmente nesse período ocorre a introdução de comidas sólidas. Uma vez que as comidas sólidas são introduzidas, o tipo de dieta tem grande reflexo na proporção de *Firmicutes/Bacteroidetes* e ácidos graxos de cadeia curta produzidos (De Filippo et al. 2010). Dentre os muitos componentes

dietéticos, hipotetiza-se que dietas ricas em amido, oriundo de arroz, batata e grãos refinados predisponham a uma maior quantidade de *Firmicutes* e *Actinobacteria*, e a uma menor quantidade de *Bacteroidetes*, mas mesmo nessas dietas há o impacto do consumo e fonte de fibras oriundas de grãos integrais e vegetais (Jain et al. 2018). O microbioma responde de forma rápida a alterações na dieta, sobretudo quando há uma mudança nas quantidades de polissacarídeos de origem vegetal, alterando principalmente os níveis de *Firmicutes* (David et al. 2014b).

Os carboidratos são as fontes centrais para a fermentação microbiana intestinal, e podem ser obtidos através de diferentes fontes: glicanos oriundos da dieta ou derivados do hospedeiro (como a mucosa colônica), sintetizados por outros micróbios que são de origem alimentar, como a parede celular de leveduras ou de outros residentes intestinais ou, ainda, de material vegetal, como as fibras dietéticas, as quais são a fonte mais comum para a microbiota de muitos seres humanos (Sonnenburg and Sonnenburg 2014). O consumo de fibras (principalmente envolvendo frutanos e galacto-oligossacarídeos) afeta diretamente a microbiota e está associado ao aumento da abundância fecal dos gêneros *Bifidobacterium* (*Actinobacteria*) e *Lactobacillus spp*. (*Firmicutes*), e ao aumento da produção de butirato, sem interferir na alfa-diversidade (So et al. 2018).

Dietas pobres em fibras, tais como a dieta ocidental típica, estão diretamente associadas à degradação da barreira mucosa colônica, uma defesa primária contra patógenos entéricos (Desai et al. 2016). As dietas ocidentais (caracterizadas por um alto consumo de gorduras animais, principalmente ácido graxo poli-insaturados ω-6, açúcares refinados e carne, e baixo consumo de frutas e vegetais) são pobres em carboidratos acessíveis à microbiota e ricas em alimentos ultraprocessados e aditivos alimentares, os quais estão relacionados à ruptura do equilíbrio intestinal e ao desenvolvimento de doenças metabólicas e inflamatórias, como diabetes, obesidade (Zinöcker and Lindseth 2018) e doenças inflamatórias intestinais (Limdi 2018). Embora a maior parte dos estudos associem a dieta a doenças tradicionalmente ligadas aos hábitos alimentares e ao trato digestivo, a ruptura do equilíbrio microbiano via alimentação tem

associações com doenças e microbiomas em sítios distais, tais como doenças alérgicas das vias aéreas, alterações na hematopoiese (Trompette et al. 2014), câncer de mama e alteração do microbioma das glândulas mamárias (Shively et al. 2018).

Além do tipo e da composição dos carboidratos, o consumo de vitaminas, principalmente as pertencentes ao grupo B e folato têm o potencial de alterar a microbiota intestinal (Maynard et al. 2018; Gurwara et al. 2019). Apesar de alguns grupos bacterianos serem capazes de sintetizar as principais vitaminas necessárias ao seu metabolismo de forma total ou parcial (Magnúsdóttir et al. 2015), a comunidade microbiana como um todo compete com o hospedeiro pelas vitaminas de síntese microbiana mais restrita, como a vitamina B_{12}, por exemplo (Degnan et al. 2014). A vitamina B_{12} consumida pelo hospedeiro é apenas parcialmente absorvida no intestino delgado, e o excedente é utilizado, em parte, pela comunidade bacteriana. Essa disponibilidade de vitaminas produzidas apenas por poucos membros da microbiota atua como modulador para as populações não produtoras (Magnúsdóttir et al. 2015), e, dependendo da quantidade, pode estar envolvida em processos de patogênese microbiana (Rowley and Kendall 2019).

2.1.1.2 A genética e o microbioma intestinal

A composição da microbiota intestinal é mais influenciada pela proximidade genética entre duas populações, enquanto a abundância relativa sofre uma influência maior de fatores geográficos (Hansen et al. 2019). Pesquisas com gêmeos mono e dizigóticos demonstram que existem taxas influenciados pela genética do hospedeiro, mas que essa herdabilidade é limitada (Goodrich et al. 2014). Os genes que possuem maior influência sobre a microbiota intestinal são aqueles ligados ao metabolismo e ao sistema imune (Dąbrowska and Witkiewicz 2016).

Em uma expansão do estudo original realizado em 2014, Goodrich e colaboradores (2016) identificaram e confirmaram que diversos genes e bactérias estão correlacionados. A família *Christensenellaceae* permaneceu como o taxa

mais influenciado pela genética do hospedeiro, incluindo o gênero *Metanobrevibacter*. Este gênero é particularmente interessante por apresentar uma correlação positiva com uma alfa diversidade maior. Uma outra importante associação é entre *Bifidobacterium* e o gene *LCT* (lactase), de forma que em pessoas cuja lactase deixa de ser produzida após o desmame possuem mais *Bifidobacterium* do que aquelas que são lactase-persistentes (Goodrich et al. 2016).

Algumas relações podem ser explicadas pelo aporte de substrato para as reações de fermentação bacterianas, como é o caso de *Bifidobacteria* e da lactose, acima descrito. Outras, no entanto, possuem um mecanismo mais complexo, como no caso dos genes que afetam o sistema imune e participam do reconhecimento do próprio e não-próprio, e dos *loci* de herança quantitativa.

Recentemente, verificou-se que parte do efeito das variantes genéticas que elevam o risco para doença inflamatória intestinal provavelmente são mediados por um efeito sobre a microbiota (Aschard et al. 2019). Entretanto, evidências de que genética e o microbioma estão intimamente relacionados existem em estudos mais antigos, como, por exemplo, na associação entre loci genômicos que predispõem à obesidade e à plasticidade de taxa bacterianos em resposta a diferentes dietas (Parks et al. 2013). Estudos como este sugerem que a manipulação bacteriana intestinal pode ser uma terapia efetiva para superar a suscetibilidade gênica à algumas doenças (Aschard et al. 2019), uma vez que as bactérias, em alguns casos, parecem modular a susceptibilidade do hospedeiro a fatores ambientais, intervenções dietéticas e doenças.

2.2 Interação entre microbioma intestinal e hospedeiro

As bactérias intestinais produzem metabólitos que agem diretamente sobre o hospedeiro, estando ligadas à síntese e absorção de várias vitaminas, transformação de ácidos biliares (Molinero et al. 2019), metabolismo de xenobióticos (Clarke et al. 2019), digestão de carboidratos não digeríveis pelo organismo humano e síntese de ácidos graxos de cadeia curta (AGCC), os quais atuam na regulação de vários processos fisiológicos do hospedeiro (den Besten et al. 2013; Molinero et al. 2019). A interação entre microbioma e hospedeiro,

entretanto, é um eixo bidirecional e ocorre tanto pela resposta bacteriana frente aos metabólitos gerados pelo hospedeiro (David et al. 2014b; Ríos-Covián et al. 2016) quanto pelas respostas fisiológicas do hospedeiro frente aos metabólitos bacterianos (Lakhan and Said 2017; Murugesan et al. 2018). A evidência é de que esses processos de interação se iniciem ainda na fase uterina, exercendo impacto sobre aspectos básicos da fisiologia humana, tais como no desenvolvimento de órgãos como o coração (Guzzardi et al. 2019). Esta intrincada relação tem levado ao surgimento do estudo de eixos bidirecionais mais específicos, tais como o eixo microbioma-cérebro-intestino (Wiley et al. 2017; Dinan and Cryan 2017; Pan et al. 2019), o eixo intestino-fígado (Hamoud et al. 2018) e, de forma mais recente, o eixo músculo-intestinal (Ticinesi et al. 2017; Grosicki et al. 2018).

A dieta do hospedeiro é determinante quanto à quantidade e qualidade de substratos para as reações metabólicas bacterianas, e pode ser encarada como um fator chave na manutenção de uma relação saudável entre microbiota e hospedeiro (Zhang et al. 2018). É pelo processo de fermentação de carboidratos não digeríveis pelos humanos que as bactérias geram os AGCC, principalmente acetato, butirato e propionato (den Besten et al. 2013). Estes metabólitos são capazes de influenciar uma miríade de reações fisiológicas no organismo humano (Roy et al. 2006; den Besten et al. 2013), sendo, em última instância, reguladores globais da acetilação de histonas e processos de metilação no cólon, fígado e tecido adiposo branco, interferindo na expressão de mais de 600 genes, com ontologias ligadas ao armazenamento lipídico, sensibilidade à insulina e sistema imune (Krautkramer et al. 2016).

O sistema imune, por sua vez, é o mecanismo de interação entre microbiota e hospedeiro mais estudado, e talvez o mais complexo. Nos últimos anos, a imunologia vem sendo revolucionada pelo crescimento no entendimento do papel fundamental da microbiota na indução, educação e função do sistema imune humano (Belkaid and Harrison 2017). Patologias como alergias, doenças inflamatórias e imunológicas, na maioria das vezes, surgem da falha no controle do sistema imune de evitar respostas danosas contra o próprio, contra antígenos ambientais ou oriundos da microbiota. Além disso, alterações na composição e função da microbiota como resultado do uso de antibióticos ou de mudanças

dietéticas alteram os padrões de interação, de forma que essa relação é contextual, e um mesmo micróbio pode ter uma relação de mutualismo ou de parasitismo, de acordo com o estado nutricional, co-infecção ou panorama genético do hospedeiro (Belkaid and Hand 2014).

A manutenção da homeostase entre hospedeiro e microbiota deve-se, em grande parte, à integridade da barreira intestinal. Composta por células epiteliais, muco, imunoglobulina A e peptídeos antimicrobianos, a barreira intestinal minimiza o contato entre microrganismos e células do epitélio, limitando a inflamação tecidual e a translocação de bactérias intestinais. As bactérias, por sua vez, secretam AGCC, os quais inibem a ativação de neutrófilos. Além disso, o butirato é um potente modulador entérico da atividade de células T (Belkaid and Hand 2014). Uma vez rompida a homeostase do sistema, as consequências poderão ser locais ou sistêmicas, afetando os mais diversos órgãos. O fígado é um dos órgãos mais afetados, uma vez que 80% do sangue circulante neste órgão é oriundo da veia porta, a qual drena intestino, baço e pâncreas (Belkaid and Naik 2013). Então, apesar de não estar em contato direto com as bactérias intestinais, a função imune hepática é condicionada pela exposição constante a ligantes ou metabólitos dela originados, e o fígado atua como um sensor de alterações súbitas na microbiota, traduzindo essas mudanças para o sistema metabólico e imunológico (Crispe 2009).

Recentemente, houve a descrição de que metabólitos bacterianos seriam capazes de interagir diretamente com organelas celulares, sendo capazes de modular processos mitocondriais (Han et al. 2019). Sabe-se que processos de fissão e fusão mitocondrial são, pelo menos parcialmente, controlados pela disponibilidade de nutrientes e que respondem a sinais metabólicos celulares. Ainda que utilizando modelo simplificado de interação, essa hipótese tem por base a descrição de respostas mitocondriais a betaína, metionina e homocisteína bacterianas, as quais possuem a capacidade de regular a homeostase do depósito lipídico em *Caenorhabditis elegans* (Lin and Wang 2017)*;* e também a resposta mitocondrial ao ácido colânico neste mesmo modelo experimental. O ácido colânico bacteriano, após sofrer endocitose pelas células intestinais, aumenta os processos de fragmentação mitocondrial e de repostas a proteínas

mal dobradas (UPR^{mt}) por influência gênica (via DRP-1 e ATFS-1, respectivamente), causando um aumento no tempo de vida e protegendo conta patologias associadas à idade, como progressão de tumores de células germinativas e acumulação tóxica de proteínas beta-amiloide em *C. elegans* (Han et al. 2017).

De forma geral, com o estudo das comunidades microbianas independente de cultura tornou-se possível avaliar a real dimensão da riqueza microbiana que o corpo humano abriga, o valor e a extensão de suas interações. Ainda que atualmente estejamos muito aquém da completa compreensão dos seus mecanismos de ação e modos de interação, já é possível vislumbrar o amplo espectro de sua influência nos estados de saúde e doença humanos, e o potencial terapêutico que ela abriga (Barrea et al. 2019). Não por acaso, há um crescente corpo de estudos envolvendo transplante fecal (Cold et al. 2019; Tariq et al. 2019), uso de pré e probióticos (Suzumura et al. 2019), e mecanismos de ação de medicamentos já conhecidos sobre o microbioma (Greenhill 2019; Liao et al. 2019).

2.3 Erros Inatos do Metabolismo

Os erros inatos do metabolismo (EIM) são um conjunto heterogêneo de desordens genéticas, herdadas ou oriundas de mutações espontâneas (Jeanmonod and Jeanmonod 2019), causadas por mutações em genes que codificam proteínas ou enzimas com função metabólica, causando a disfunção de uma via. A maioria dos EIM é herdada de forma autossômica recessiva, embora existam formas autossômicas dominantes e desordens ligadas ao X. A clínica dos EIM surge da acumulação do substrato tóxico, deficiência do produto ou de ambos. Normalmente a doença se manifesta ainda na infância, mas dependendo da atividade residual da enzima o aparecimento da clínica pode ocorrer somente na idade adulta (Ezgu 2016).

Ainda que um EIM seja caracterizado por um único gene defeituoso, normalmente há presença de um espectro fenotípico dentro de uma mesma doença, não havendo relação clara entre genótipo e fenótipo para a maior parte

dos casos. Este fato acabou lançando as bases para o estudo dos EIM como desordens complexas, onde fatores ambientais, tais como alimentação, exercícios, epigenética e microbioma podem exercer influência sobre o fenótipo apresentado (Argmann et al. 2016).

Os EIM são agrupados em grandes classes, dependendo do tipo de metabolismo envolvido. O agrupamento clássico compreende quatro classes gerais: doenças no metabolismo de carboidratos, metabolismo de aminoácidos, metabolismo de ácidos orgânicos e doenças de depósito lisossômico (Das 2013). Entretanto, uma classificação mais específica, como a realizada pela "Society for the Studies of Inborn Errors of Metabolism" (SSIEM*)*, categoriza os EIM hierarquicamente em 15 grandes classes, posteriormente subclassificadas, onde os EIM nos grupos pertencem à mesma via metabólica, possuem características clínicas similares, são detectados pelo mesmo procedimento diagnóstico e tratados por princípios similares de intervenção de emergência e manejo em longo prazo (SSIEM).

As estratégias de tratamento dos EIM variam conforme a doença, e podem ser realizadas com dietas especiais, terapia de reposição enzimática, inibição do substrato ou transplante de órgãos, cada qual utilizado isoladamente ou em conjunto (Schwartz et al. 2008).

2.3.1 Doenças do metabolismo de carboidratos: as glicogenoses

As glicogenoses (GSD) são um grupo de doenças pertencentes à classe de desordens do metabolismo de carboidratos envolvidos nas vias de síntese e degradação do glicogênio. Atualmente, existem 20 tipos de GSD pela classificação do SSIEM. Os órgãos mais afetados são o fígado, músculo esquelético, coração e, algumas vezes, o sistema nervoso central; mas pode haver envolvimento de diferentes órgãos, de forma que a classificação depende não só da deficiência enzimática envolvida, mas também dos órgãos afetados (Vega et al. 2016).

Em uma classificação prática, as GSD podem ser divididas entre aquelas de manifestação majoritariamente hepáticas ou musculares. Nas GSD hepáticas (tipos 0, I, III, IV, VI, IX e XI), a manifestação primária é a hipoglicemia ao jejum. É importante ressaltar que na forma clássica de GSD tipo IV, só há hipoglicemia após a falência hepática, ocasionada por cirrose (Kishnani et al. 2014; Weinstein et al. 2018). Já nas GSD classificadas como musculares (tipos II, III, IV, V, VII e X), a manifestação primária é a presença de fraqueza muscular e/ou de câimbras musculares. A GSD tipo III é a única com características hepáticas e musculares de forma concomitante (Weinstein et al. 2018). As manifestações clínicas e o perfil laboratorial dependerão das vias afetadas, tanto pela ausência da proteína quanto pelas rotas compensatórias utilizadas pelo organismo. Detalhes sobre este tópico serão feitos na seção a seguir, apenas para as GSD de interesse nesta tese.

2.3.2 As glicogenoses hepáticas e seu tratamento

A glicose é a principal forma de energia circulante na corrente sanguínea. A maior parte dos processos de obtenção de energia celular residem na difusão da glicose através de membranas celulares. A difusão é facilitada por transportadores (GLUT), e uma vez dentro da célula, a glicose é fosforilada à glicose-6-fosfato (G6P), o que a impede de sair. Em órgãos responsáveis pela normoglicemia (fígado, rins e em menor grau, intestino), em condições de jejum, a G6P é formada a partir da quebra do glicogênio ou da formação de glicose a partir de outros precursores metabólicos (glicogenólise e gliconeogênese, respectivamente). A G6P formada por estas vias é processada pela enzima de retículo multimérica glicose-6-fosfatase (G6Pase), formando glicose e fosfato. A glicose recém-formada retorna ao citoplasma celular via transportador e é liberada na corrente sanguínea, mantendo os níveis de glicose circulante.

Como característica em comum, todas as GSD hepáticas acumulam glicogênio no fígado e há ocorrência de hipoglicemia ao jejum. Dentre as glicogenoses hepáticas, os tipos mais comumente diagnosticados são o I, III e IX (Ozen 2007; Hicks et al. 2011). Defeitos na rota de degradação do glicogênio para

as GSD de interesse nesta tese estão representados na figura 1 e serão descritos de forma detalhada abaixo.

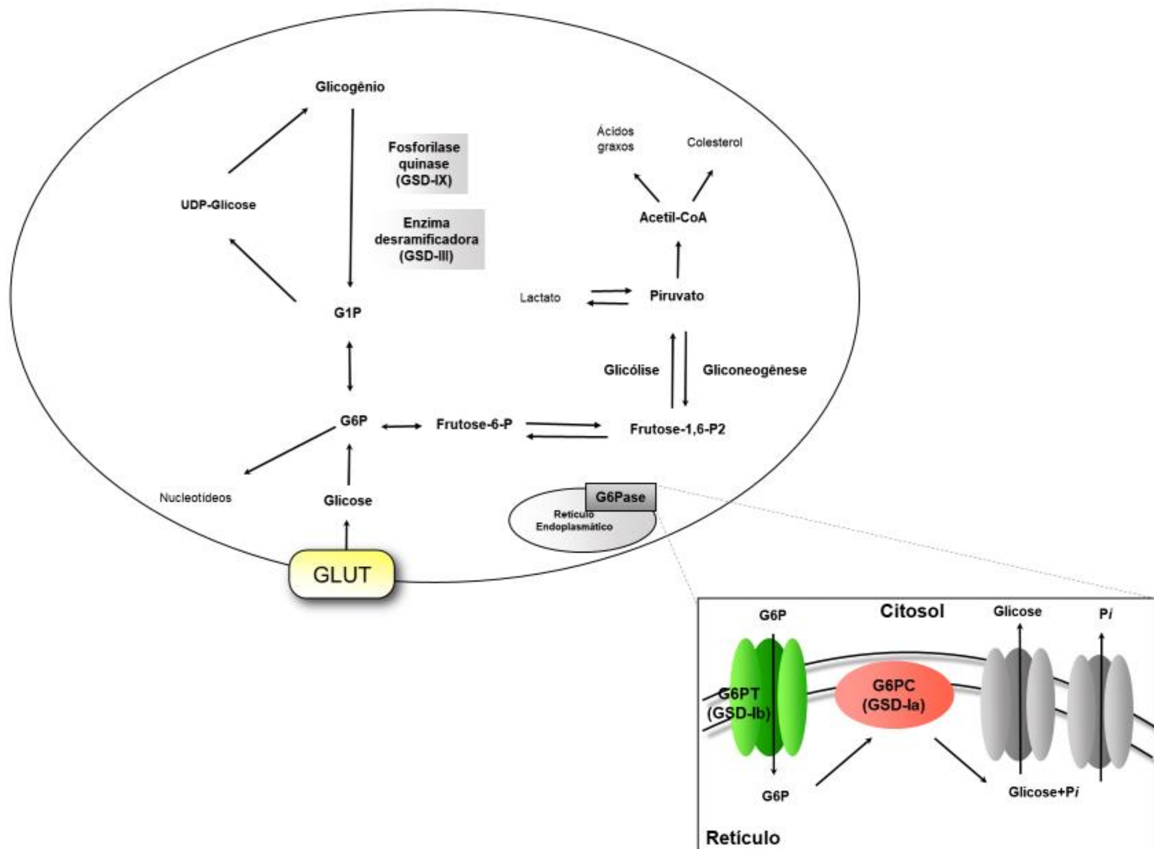

Figura 1. Representação das glicogenoses tipo Ia, Ib, III e IX e etapas metabólicas comprometidas. G6P: glicose-6-fosfato; G1P: glicose-1-fosfato; Frutose-1,6-P2: Frutose-1,6-bisfosfato; G6Pase: glicose-6-fosfatase; G6PT: transportador de glicose-6-fosfato; G6PC: subunidade catalítica 1 de glicose-6-fosfato. GSD: glicogenose. Fonte: modificado de Yang Chou e Mansfield 1999.

A GSD tipo I (GSD-I) subdivide-se em GSD-Ia e GSD-Ib, e ambos são disfunções da G6Pase. Esta enzima é, na verdade, uma proteína integral de membrana complexa, composta por subunidades catalíticas para seu substrato (G6P) e transportadores para seus produtos (glicose e fosfato inorgânico).

As subunidades catalíticas são codificadas por três genes: *G6PC*, *G6PC2* e *G6PC3*. O gene *G6PC* causa a GSD-Ia, a qual corresponde a cerca de 80% dos

casos de GSD do tipo I (Janecke et al. 2001), é majoritariamente expresso nos tecidos hepático, renal e intestino delgado, mas também é expresso, ainda que em baixos níveis, no duodeno e na vesícula biliar (NCBI, Gene ID: 2538). Já a subunidade codificada pelo gene *G6PC2* é expressa nas ilhotas pancreáticas (ilhotas de Langerhans), e em menor grau nos testículos, estômago e duodeno. Até o momento, não há atividade de fosfo-hidrolase associada à essa subunidade, mas ela é um alvo importante na diabetes quando há autoimunidade mediada por células. Embora vários transcritos alternativos tenham sido descritos, a sua validade biológica ainda não foi determinada (NCBI, Gene ID: 57818). Enquanto a expressão do *G6PC2* é localizada, a expressão do gene *G6PC3* é ubíqua, com grande atividade catalítica associada. Mutações de caráter recessivo nesta subunidade causam neutropenia congênita severa (NCBI, Gene ID: 92579).

Defeitos na subunidade transportadora de G6P da G6Pase causam a GSD-Ib. Esta subunidade é codificada pelo gene *SLC37A4,* e defeitos que a afetam inviabilizam a atividade de todas as subunidades catalíticas. Por esta razão, além dos sintomas da GSD-Ia, alguns sintomas são tipicamente atribuídos à GSD-Ib, oriundos da disfunção imunológica causada pelo bloqueio da atividade das outras subunidades (Kim et al. 2006), como a presença de neutropenia contínua ou intermitente, doença inflamatória intestinal e infecções recorrentes (Visser et al. 2012). A neutropenia da GSD-Ib é caracterizada pela apoptose aumentada dos neutrófilos (Kuijpers et al. 2003) e stress do retículo, com a contagem de neutrófilos no sangue periférico sendo inversamente proporcional ao grau de inflamação (Visser et al. 2012). Nestes pacientes, a administração de G-CSF é utilizada para aumentar a contagem de neutrófilos (Ishiguro et al. 1993; Calderwood et al. 2001), e, embora não aumente a quimiotaxia celular (McCawley et al. 1994), em alguns casos, foi capaz de resolver a doença inflamatória intestinal (Alsultan et al. 2010)

A GSD tipo III (GSD-III) é causada por deficiência da enzima desramificadora do glicogênio (*AGL*). Na GSD tipo III, parte do glicogênio é quebrado normalmente. A gliconeogênese não é afetada, e exibe uma atividade compensatória, utilizando energia de ácidos graxos e outros precursores (Chen and Weinstein 2016). Portanto, o acúmulo hepático é menor e a hipoglicemia

geralmente é menos severa que aqueles causados pela GSD-I, apesar de haver risco de cirrose, adenomas e carcinomas (Sentner et al. 2016), mesmo em pacientes assintomáticos (Oterdoom et al. 2015).

O tipo IX é causado pela deficiência da enzima fosforilase quinase, uma enzima multimérica formada por quatro subunidades (α, β, γ e δ), cada qual codificada por um ou mais genes. O gene afetado determinará se a deficiência será hepática, muscular ou se afetará os dois tecidos, e também o padrão de herança da doença. Os genes *PHK*A1 e *PHKA2* codificam para a subunidade α, com modo de herança ligado ao X. Defeitos em *PHK1* levam à manifestação muscular da doença, enquanto defeitos em *PHK2* possuem manifestação hepática e são o tipo mais comum de GSD-IX. Os outros dois genes são herdados de modo autossômico recessivo. O gene *PHKB* codifica para a subunidade β, e causa deficiência hepática e muscular. Um terceiro gene, *PHKG2*, codifica para a subunidade γ e causa a deficiência no tecido hepático. Mutações nos genes que codificam para a subunidade δ não são conhecidas (Herbert et al. 1993). De maneira geral, o tratamento das GSD hepáticas busca eliminar a ocorrência de hipoglicemias, e prevenir distúrbios metabólicos secundários e o depósito de glicogênio no fígado através de restrição de carboidratos de hidrólise rápida e da administração frequente e periódica de amido de milho cru (AMC).

A ingestão de AMC foi introduzida em 1984, a fim de tratar a hipoglicemia em pacientes de GSD-I (Chen et al. 1984), mas hoje é recomendada também para os outros tipos de GSD hepáticas (Chen and Weinstein 2016). A quantidade de AMC ingerida varia conforme a idade e o peso do paciente, sendo altamente personalizado. Ajustes são feitos com base no relato dos pacientes, atividades físicas praticadas, e avaliação rigorosa do controle metabólico, baseado em medidas de glicemia diárias (Kishnani et al. 2014). Nos tipos de GSD em que a gliconeogênese está intacta, as restrições dietéticas são menos severas, e uma dieta hiperproteica também é recomendada (Chen and Weinstein 2016). As principais manifestações clínicas e linhas gerais de tratamento para as GSD supracitadas podem ser encontradas na Tabela 1.

Tabela 1. Principais características das glicogenoses (GSD) hepáticas de interesse nesta tese.

Tipo de GSD (OMIM#)	Proteína afetada	Gene (modo de herança)	Principais manifestações clínicas	Linhas gerais de tratamento
GSD-Ia (232200)	Glicose-6-fosfatase (subunidade catalítica)	*G6PC* (AR)	Hipoglicemia ao jejum, hepatomegalia, retardo no crescimento, hiperlipidemia. Pode haver: densidade mineral óssea reduzida, anemia, adenoma hepático, sobrepeso, síndrome metabólica, disfunção plaquetária e disfunção renal, hipertensão pulmonar e sistêmica, deficiência de vitamina D	AMC Restrição de galactose, frutose, lactose e sacarose Alopurinol Citrato
GSD-Ib (232220)	Glicose-6-Fosfato translocase	*SLC37A4* (AR)	Similar à Ia, mais neutropenia, infecções recorrentes e doença inflamatória intestinal, disfunção da tireoide (autoimune e hipotireoidismo)	AMC Restrição de galactose, frutose, lactose e sacarose G-CSF Mesalazina
GSD-III (232400)	Enzima desramificadora do glicogênico	*AGL* (AR)	Hipoglicemia ao jejum, hepatomegalia, retardo no crescimento, hiperlipidemia, cirrose hepática, carcinoma hepatocelular	AMC Dieta hiperproteica Restrição de sacarose
GSD-IXα (306000)	Fosforilase quinase (subunidade α)	*PHKA2* (XL)	Hipoglicemia ao jejum, hepatomegalia, retardo no crescimento, hiperlipidemia leve	AMC Dieta hiperproteica Evitar grandes quantidades de sacarose
GSD-IX*c* (613027)	Fosforilase quinase (subunidade γ)	*PHKG2* (AR)	Hipoglicemia ao jejum, hepatomegalia, retardo no crescimento, hiperlipidemia leve	AMC Dieta hiperproteica Evitar grandes quantidades de sacarose

AR: autossômico recessivo; XL: ligado ao X; AMC: amido de milho cru; G-CSF: fator estimulante de granulócitos. Fonte: OMIM, Kishnani et al. 2014; Sentner et al. 2016; Chen and Weinstein 2016.

O uso do AMC é o tratamento mais comum e presente nas diretrizes de manejo; entretanto, existem outras formas de terapias dependendo do tipo de glicogenose e das comorbidades presentes, tais como o uso de amidos modificados (Correia et al. 2008; Ross et al. 2015) (ClinicalTrials ID:

NCT02318966), dieta cetogênica (Valayannopoulos et al. 2011; Brambilla et al. 2014) e transplante de órgãos (Marega et al. 2011; Maheshwari et al. 2012; Choi et al. 2016; Zobeiri 2017). Além destas, estão em fase experimental alternativas terapêuticas como a utilização de terapia gênica (ClinicalTrials ID: NCT03517085), o uso do polvilho doce como fonte de amido (ClinicalTrials ID: NCT03871673) e a reposição de anapleróticos do ciclo de Krebs (ClinicalTrials ID: NCT03665636).

2.4 Estudos sobre microbiota e microbioma nas glicogenoses

Os estudos existentes sobre microbiota e microbioma nas GSD são poucos. Buscas nas bases de dados do PubMed e Google Acadêmico retornam apenas 3 artigos, todos nos últimos 5 anos. Um breve resumo de cada um deles pode ser encontrado abaixo.

O estudo mais robusto quanto à microbiota nestes pacientes é odontológico e compreende a análise da microbiota oral, onde 53 pacientes (Ia=11, Ib=02, III=04, IX=12) tiveram placas supragingivais e subgingivais coletadas e foram avaliados em relação às espécies cariogênicas e peripatogênicas por sequenciamento da região V3-V4 do gene *16S rRNA* e PCR quantitativo. Pacientes alimentados por via oral apresentaram médias maiores de espécies cariogênicas e espécies associadas à saúde periodontal, enquanto os pacientes alimentados por tubos de gastrostomia apresentaram maiores níveis de peripatógenos. A hipótese é de que o amido, uma vez que libera glicose lentamente, ajuda na formação de biofilmes na superfície dental. Entretanto, ingestão frequente de glicose e seu posterior metabolismo nos biofilmes orais resultariam na produção de ácido, o que leva à desmineralização do esmalte e ao posterior desenvolvimento de cáries dentais (Garcia et al. 2016).

Os outros dois estudos são relatos de caso. O primeiro deles descreve o uso do probiótico comercial VSL#3 em um paciente de 36 anos com GSD-Ia diagnosticado com doença inflamatória intestinal (Crohn-like) por endoscopia e biópsia intestinal. O probiótico utilizado é um dos probióticos comerciais mais estudados até o momento. Por quatro semanas o paciente foi tratado com uma

unidade do probiótico a cada 12h (2/24h), seguido por um período de 72 horas sem tratamento. Após as 72 horas, por uma semana administrou-se novamente duas unidades a cada 12 horas (2/24h), e na semana seguinte, uma unidade do probiótico por dia por mais uma semana. O probiótico, quando utilizado a cada 12 horas, foi associado à redução dos episódios de diarreia e dor abdominal, bem como à melhor absorção de nutrientes e redução de importantes marcadores clínicos da glicogenose em questão. Um aumento de gama glutamil transferase foi relatado (Carnero-Gregorio et al. 2018).

O outro relato de caso diz respeito a uma paciente tratada com transplante fecal. A paciente, de 21 meses de idade, possuía diagnóstico de GSD-II (maltase ácida, lisossômica, OMIM #232300) e imunodeficiência de células B, com infecção recorrente por *Clostridium difficile* refratária a antibioticoterapia. A mãe doou 11g de fezes para o transplante, e, após o procedimento, houve resolução dos sintomas e cura sustentada por 5 anos (até a publicação do estudo) (Dow and Seed 2018).

2.5 Estudos sobre microbioma e manifestações secundárias das glicogenoses hepáticas

A maior parte dos pacientes com glicogenoses hepáticas diagnosticados são do tipo I. Estes pacientes possuem uma tendência ao sobrepeso e à obesidade, cuja etiologia ainda não é completamente esclarecida (dos Santos et al. 2017). Além disso, pacientes com GSD-Ia possuem um risco aumentado para o desenvolvimento de doença óssea (Minarich et al. 2013), síndrome metabólica (Melis et al. 2015) e surgimento de adenoma hepatocelular, os quais podem sofrer malignização (Franco et al. 2005; Cho et al. 2018) e necessitar de transplantes (Faivre et al. 1999). Os pacientes do tipo Ib, por sua vez, além da obesidade, possuem risco aumentado para doença inflamatória intestinal (Visser et al. 2000; Melis et al. 2003) e doenças autoimunes de tireóide (Melis et al. 2007; Melis et al. 2017). Os tipos III e o tipo IX são metabolicamente menos comprometidos, mas também existe o risco de doença hepática (Leuzinger Dias et al. 2019) com cirrose e posterior evolução para carcinoma hepatocelular (Demo et al. 2007).

A obesidade está associada a múltiplas patologias, incluindo síndrome metabólica, doenças cardiovasculares e câncer (Pi-Sunyer 2002). O microbioma intestinal atua como um fator ambiental no desenvolvimento da obesidade, pois a microbiota interfere em processos fisiológicos, como digestão e metabolismo, podendo aumentar a produção de energia oriunda da dieta e modular a composição dos tecidos em relação ao seu teor de ácidos graxos, além de gerar inflamação em baixo grau, a qual está envolvida não só na obesidade como em outras desordens metabólicas (Cani et al. 2012).

Relações causais entre microbiota e síndrome metabólica tiveram início quando um receptor de transplante fecal com síndrome metabólica experimentou melhora na sensibilidade à insulina após receber fezes de um doador magro. O transplante gerou um aumento da diversidade e quantidade de butirato nas fezes, com um aumento da abundância relativa de bactérias produtoras de butirato, como *Roseburia intestinalis* (Vrieze et al. 2012). Recentemente, um estudo de associação com 950 participantes normoglicêmicos relacionou os dados genômicos dos indivíduos, dados metagenômicos intestinais e os níveis fecais de ácidos graxos de cadeia curta com 17 traços metabólicos e antropométricos apresentados pelos participantes. Após validação em biobanco com mais de 500.000 amostras, houve associação de traços genéticos do hospedeiro, aumento na produção intestinal de butirato, e resposta aumentada à insulina após teste de tolerância oral à glicose. Além disso, detectou-se que anormalidades na produção ou absorção de propionato estavam associados de forma causal ao risco aumentado de desenvolvimento de diabetes tipo 2, fornecendo evidência do efeito do microbioma intestinal sobre as características metabólicas (Sanna et al. 2019).

A descrição de alteração da microbiota intestinal em pacientes com doenças hepática crônica é antiga, e o primeiro caso foi descrito cerca de 80 anos atrás (Yu et al. 2016), de forma que atualmente existe uma série de dados, ainda em caráter emergente, associando a microbiota intestinal com esteato-hepatite não-alcóolica (EHNA). Apesar do mecanismo não ser completamente compreendido, inflamação, dano à barreira intestinal, translocação bacteriana e endotoxemia, tem sido sugeridos como possíveis mecanismos (Farhadi et al. 2008), assim como uma mudança no perfil metabólico das bactérias intestinais

(Zhu et al. 2013). Não só a presença de EHNA tem relação com a microbiota, uma vez que *Bacteroides* são associados com a patologia de forma geral, mas também a severidade e progressão da doença, havendo associação de *Ruminococcus* com estados de fibrose (Boursier et al. 2016). Uma das possíveis complicações da doença hepática, além cirrose e fibrose, é a malignização das lesões, as quais darão origem ao carcinoma hepatocelular. A disbiose intestinal está associada a todas as complicações (Jiang et al. 2019), e foi capaz de promover a progressão para HCC em modelos animais, onde o receptor do tipo Toll-4 (TLR4) parece ter envolvimento central (Dapito et al. 2012), sendo inclusive sugerido como alvo terapêutico (Darnaud et al. 2013).

Em pacientes que realizaram transplante hepático, foi descrito uma alteração significativa da microbiota após o transplante, com diminuição de gêneros como *Actinobacillus*, *Escherichia* e *Shigella*, e um aumento de outros taxa, como *Micromonosporaceae*, *Desulfobacterales* e dos gêneros *Sarcina* (*Eubacteriaceae*), e *Akkermansia* (Sun et al. 2017). Alterações funcionais e metabólicas relacionas ao microbioma também já foram descritas em pacientes transplantados (Bajaj et al. 2018). Ainda no contexto dos transplantes, vários fatores pré, intra e/ou pós transplante podem resultar em disbiose, incluindo o uso de antibióticos, imunossupressores e quimioterapia, além da anatomia pós-cirúrgica (que pode causar alterações anatômicas, funcionais e/ou neuromusculares). A disbiose pode levar a (ou atuar como modificador em) algumas complicações pós-cirurgicas, como risco de infecções (infecção urinária, diarreia infecciosa), fenômenos imunológicos adversos (anemia hemolítica auto-imune), rejeição do transplante, e ao aumento das taxas de mortalidade (Tabibian and Kenderian 2017).

A presença de IBD é um achado comum em pacientes com GSD-Ib e tem sido descrita de forma cada vez mais frequente em pacientes com GSD-Ia. A etiologia da IBD é em parte atribuída a disbiose intestinal. Como traço comum aos diferentes tipos de IBD, sabe-se que o microbioma intestinal destes indivíduos é mais instável, variando mais do que o microbioma de pessoas saudáveis (Halfvarson et al. 2017). Os ácidos graxos oriundos da fermentação bacteriana também são associados ao desenvolvimento de IBD. Vários estudos relatam que

há uma diminuição nos níveis de AGCC na mucosa e nas fezes de pacientes com doença inflamatória intestinal, e eles desempenham papéis cruciais na manutenção da homeostase entérica, seja através da manutenção da função da barreira intestinal, na geração de energia nos colonócitos, ou exercendo seu papel de imunomodulação através dos receptores acoplados de proteína G (GPCRs), os quais controlam a inflamação intestinal (Parada Venegas et al. 2019). Tanto o perfil microbiano fecal quanto a predição funcional do microbioma possuem um valor terapêutico, uma vez que existe associação entre perfis microbianos fecais e atividade da doença (Tedjo et al. 2016); e também entre as vias funcionais bacterianas e resposta ao tratamento para IBD (remissão) (Ananthakrishnan et al. 2017).

As doenças ósseas em GSD são normalmente aquelas relacionas à diminuição da densidade mineral óssea, causando osteopenia ou osteoporose (Minarich et al. 2013). O microbioma desempenha um papel importante na indução de osteoclastos em modelos animais, interage com o sistema imune e participa dos processos de inflamação do organismo através da absorção de vitamina D, indução da atividade de células B e T, liberação de citocinas (IL-17A e IL-6), transformação de ácidos biliares, e produção de AGCC (D'Amelio and Sassi 2018; Li et al.) Atualmente, os estudos em humanos começam a ganhar forma, confirmando associações entre disbiose e densidade mineral óssea reduzida (Li et al. 2019). O uso de probióticos tem sido associado à um aumento da densidade mineral óssea, e um dos mecanismos propostos é a indução da expressão de proteínas das *tight junction* intestinais, o que aumentaria a força do epitélio e melhoraria a função de barreira, diminuindo a apresentação de antígenos e respostas imunes associadas (Hsu and Pacifici 2018).

2.6 Estudos sobre citocinas nas glicogenoses hepáticas

As citocinas controlam virtualmente todos os aspectos do organismo humano, influenciando os mais diversos aspectos fisiológicos, tais como crescimento, adiposidade, lactação, hematopoiese, além dos processos inflamatórios e imunes de forma geral (Braunersreuther et al. 2012; Gorby et al.

2018). As citocinas são uma ampla classe de proteínas solúveis, compreendendo várias subfamílias, tais como interferons (INF), interleucinas (IL), fatores de necrose tumoral (TNF), fatores de crescimento (TGF), fatores estimulantes de colônias (CSF) e quimiocinas (Cameron and Kelvin 2013). Uma classificação simplista é normalmente adotada, com a divisão entre citocinas pró (IL-1, IL-6, TNF, IFN-γ, IL-12, IL-18 e GM-CSF) e anti-inflamatórias (IL4, IL-10, IL-13, IFN-α, TGF-β). Entretanto, esta classificação dual é equivocada e serve apenas como referencial, não representando, de fato, a complexidade dos eventos biológicos *in vivo* (Cavaillon 2001; Conti et al. 2003; Scheller et al. 2011; Schett 2018).

Dois estudos analisaram as citocinas em camundongos *knockout* modelos para a GSD-Ia e GSD-Ib. O estudo mais antigo, realizado com animais *knockout* para a subunidade transportadora da G6Pase (GSD-Ib), demonstrou que os camundongos desenvolviam neutropenia, apresentavam níveis maiores de fator estimulante de colônia de granulócitos (G-CSF) e de fator quimioatraente de neutrófilos induzido por citocinas (KC, um análogo murinho da IL-8 humana) em comparação ao grupo controle (Chen et al. 2003). Essas alterações foram confirmadas no plasma de pacientes com GSD-Ib que não estavam em tratamento com G-CSF. Quando um experimento similar foi realizado com animais *knockout* para a subunidade catalítica da G6Pase (GSD-Ia), resultados similares foram verificados, embora o aumento de G-CSF e KC tenha sido mais discreto nestes animais (Kim et al. 2007). Em um estudo posterior, com 55 pacientes (idade 2-39 anos) metabolicamente compensados e 28 controles pareados por idade, pacientes com GSD-Ia exibiram neutrofilia em comparação ao grupo controle. Neste estudo, os indivíduos com adenomas apresentaram maior porcentagem de neutrófilos no sangue em comparação aos pacientes sem adenomas. Ainda, o nível de IL-8 foi maior nos pacientes com GSD-Ia do que nos controles, e os níveis de IL-8 nos pacientes com adenoma foram em média duas vezes maiores do que nos pacientes sem adenomas. Os níveis de G-CSF não variaram entre os grupos (Kim et al. 2008).

Apesar da neutropenia clássica descrita para pacientes com GSD-Ib, há um breve relato na literatura informando sobre um paciente de 10 anos de idade com GSD-Ib, neutropenia e disfunção de neutrófilos que nunca sofreu infecções

recorrentes severas. A resposta imune celular do paciente foi avaliada pelas subpopulações de linfócitos e pela produção de citocinas IL-2, IL-4 e IFN-γ. Neste caso, um padrão de ativação de linfócitos sugeriu um deslocamento da resposta Th1 (IL-2, IFN-γ)/Th2 (IL-4) favorecendo as respostas imunes do tipo Th1. Foi detectado, também, um aumento na população celular tipo CD56 (células Natural Killer), com uma relação normal das populações celulares T-CD4/T-CD8 (linfócitos T auxiliares e linfócitos T citotóxicos, respectivamente) (D'Eufemia et al. 2007).

Em um estudo mais abrangente, Marfaing-Koka e colaboradores (2003) estudaram uma amostra de 27 pacientes com GSD-Ia (14 com adenomas), 14 pacientes com GSD-III/VI (01 paciente GSD-III com adenoma) e 30 controles saudáveis. Neste estudo, IL-6 e TNF-α foram analisados e não houve diferença entre os grupos ou diferença quanto a presença/ausência de adenomas.

3 JUSTIFICATIVA

Tendo em vista a proeminente associação entre o microbioma intestinal e as manifestações fenotípicas presentes nas glicogenoses hepáticas, e dada a falta de correlação entre genótipo e fenótipo nos pacientes, esta tese busca caracterizar a microbiota intestinal de pacientes com glicogenoses hepáticas, lançando as bases para estudos posteriores de interação e associação entre microbiota-hospedeiro que ajudem a explicar a variabilidade clínica, fatores de risco e/ou prevenção para estes pacientes.

4 HIPÓTESE

Os pacientes com glicogenoses hepáticas possuem o microbioma intestinal alterado em decorrência do uso de grandes quantidades de amido de milho cru e restrições dietéticas, sendo este um potencial amplificador dos fenótipos relacionados à inflamação e à doença de base.

5 OBJETIVOS

5.1 Objetivo Geral

Caracterizar a relação entre microbiota intestinal e erros inatos do metabolismo, com ênfase nas glicogenoses hepáticas.

5.2 Objetivos Específicos

1) Revisar a literatura existente sobre erros inatos do metabolismo e microbiota intestinal.

2) Caracterizar a microbiota intestinal de pacientes com GSD hepáticas em comparação à indivíduos controles, associando-a com dieta, fenótipos e medicamentos utilizados no tratamento.

3) Caracterizar o perfil de citocinas em pacientes com GSD hepáticas em comparação a indivíduos controle, associando-as a controle metabólico e manifestações clínicas secundárias à doença de base.

4) Avaliar possíveis modos de interação entre o microbioma intestinal, citocinas plasmáticas e calprotectina fecal no contexto da GSD-Ia, utilizando como ferramenta redes de interação proteína-proteína (IPP).

6 CAPÍTULOS

A metodologia utilizada e os resultados obtidos nesta tese serão apresentados na forma de quatro capítulos distintos, no formato de artigos científicos.

No Capítulo I encontra-se uma revisão da literatura ponderando sobre a importância do microbioma nos erros inatos do metabolismo, com base nos órgãos mais afetados pelas doenças desta categoria e a associação já descrita do microbioma com os mesmos. Aspectos sobre as formas mais comuns de tratamento nos erros inatos também são apresentadas neste capítulo.

No capítulo seguinte (Capítulo II), estudam-se os efeitos da doença genética e do tratamento com sobrecarga de amido no microbioma dos pacientes com glicogenoses hepáticas em comparação com sujeitos hígidos.

Uma vez que um dos principais modos de interação entre o microbioma e o hospedeiro é através do sistema imune, o Capítulo III versa sobre o perfil de citocinas dos pacientes com GSD, lançando as bases para a posterior associação entre citocinas e populações microbianas descritas nestes pacientes.

O Capítulo IV é um capítulo dedicado à busca de mecanismos de interação entre o microbioma intestinal com o sistema imunológico dentro do contexto genético e imunológico descrito nos capítulos anteriores nas GSD. Através da utilização da biologia de sistemas buscou-se por proteínas cujas interações são alteradas pela genética na GSD-I, suas ontologias e possíveis mecanismos de interação com o microbioma.

Os dois primeiros capítulos, já publicados, estão no formato das revistas de publicação. Os capítulos posteriores correspondem a artigos em preparação, cujas tabelas e figuras correspondentes podem ser encontradas após a seção de referências dos mesmos.

6.1 CAPÍTULO I: Microbioma e Erros Inatos do Metabolismo

Título do artigo: “The microbiome and inborn errors of metabolism: Why we should look carefully at their interplay?”

Autores: Karina Colonetti, Luiz Fernando Wurdig Roesch e Ida Vanessa Doederlein Schwartz.

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Review Article

The microbiome and inborn errors of metabolism: Why we should look carefully at their interplay?

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Abstract

Research into the influence of the microbiome on the human body has been shedding new light on diseases long known to be multifactorial, such as obesity, mood disorders, autism, and inflammatory bowel disease. Although inborn errors of metabolism (IEMs) are monogenic diseases, genotype alone is not enough to explain the wide phenotypic variability observed in patients with these conditions. Genetics and diet exert a strong influence on the microbiome, and diet is used (alone or as an adjuvant) in the treatment of many IEMs. This review will describe how the effects of the microbiome on the host can interfere with IEM phenotypes through interactions with organs such as the liver and brain, two of the structures most commonly affected by IEMs. The relationships between treatment strategies for some IEMs and the microbiome will also be addressed. Studies on the microbiome and its influence in individuals with IEMs are still incipient, but are of the utmost importance to elucidating the phenotypic variety observed in these conditions.

Keywords: Inborn errors of metabolism, microbiome, microbiota, diet, treatment.

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Introduction

The human body host a large amount of non-human genetic material, the microbiome, defined as the set of microorganisms, their genes, and the surrounding environmental conditions (Marchesi and Ravel, 2015). The human gut microbiome is believed to play an important role in the development of basic physiological systems, such as the digestive, immune, and nervous systems, and constitutes a virtual metabolic organ of unquestionable importance (Lopez-Legarrea *et al.*, 2014; Suez *et al.*, 2014; Maukonen and Saarela, 2015). The gastrointestinal (GI) tract is a metabolically rich environment that harbors approximately three-quarters of the body's immune cells, contains vagal afferent endings which respond to immune cells and immune and bacterial products (cytokines, proteases, 5-Hydroxytryptimaine and CRH for corticotropin-releasing hotmone, CRH, histamine), and has receptors for compounds produced by neuroendocrine cells (Omran and Aziz, 2014). Diet and genes related to the immune system and metabolism are among the key factors with potential to alter the bacterial community present in the gut. Thus, the associations of diet, metabolism, the central nervous system, and the immune system with the development and composition of the gut microbiome has become the object of intense interest among the scientific community (Mayer *et al.*, 2014).

Inborn errors of metabolism (IEM) are rare monogenic genetic diseases characterized by absent or deficient activity of a given enzyme and which can sometimes be managed with dietary strategies. The phenotypic heterogeneity found in IEMs is manifested mainly by the age at onset of symptoms, presence (or absence) of neurological compromise, and response to the treatment. In untreated phenylketonuria (PKU) and in propionic and methylmalonic acidemia patients, for instance, the neurological and behavior impairment are highly variable. The development of liver disease is common to several IEMs, such as tyrosinemia type 1 and urea cycle disorders. Also, the response to the treatment is not the same among patients with the same genotype.

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Convergent efforts of professionals in different fields have enabled the discovery of new mechanisms and processes whereby the microbiome can exert local and systemic effects. In this non-systematic review of the literature, we will focus on how the gut microbiome could influence the context of treatable IEMs.

The human gut microbiome

Among the various microbial habitats found in the human body, the GI tract harbors the vast majority of microbial cells (Sender *et al.*, 2016). The composition of the microbiota varies along the GI tract, both quantitatively and qualitatively, depending on the environmental conditions (pH, oxygen, etc.) (Donaldson *et al.*, 2016). In the small bowel (particularly the duodenum), the composition is similar to that of the stomach, while the large bowel (especially the colon) contains the majority of the gut's microbial population, as it is the site of fermentation, due to the availability of nutrients obtained from digestion (Madigan and Martinko, 2006).

Prior to the development of next-generation sequencing (NGS) techniques, the gene profile of these microorganisms had never been determined accurately (Grenham *et al.*, 2011). The ability to obtain a large number of gene sequences in a short period of time and at relatively low cost led to the acquisition of an immense volume of data to which biological significance could then be ascribed (Cho and Blaser, 2012). Advances in these techniques, coupled with the development of bioinformatics tools, have allowed analysis of the gut microbiome to an extent that would have been impossible with bacterial cultures alone (Hiergeist *et al.*, 2015). Furthermore, the use of NGS and bioinformatics techniques, with the aid of databases and computational and statistical algorithms, has allowed complex studies for the detection, quantification, and functional analysis of the human microbiome and its physiological associations, thus expanding knowledge of microbial ecology beyond simple pathogen *vs.* host relationships.

Initiatives such as the Human Microbiome Project, created in the United States in 2008, have sought to characterize the microbial communities of various sites in the human body, with a focus on analyzing the role of these microorganisms in sickness and in health (Human Microbiome Project Consortium, 2012). In Europe, a similar effort known as MetaHIT, which took place from 2008 to 2012, sought to study the association of the gut microbiome with several states of health and illness, prioritizing obesity and inflammatory bowel disease (Metagenomics of the Human Intestinal Tract, MetaHIT.

The results of the aforementioned initiatives have led to a new appreciation for the human microbiome from taxonomic and functional points of view. The microbiota is both functionally relevant and uniquely personal, differing even between monozygotic twins, what suggests that childhood exposure to different environmental factors is a determinant of development of the adult microbiota (Turnbaugh and Gordon, 2009). Despite great interpersonal variation in the microbiota, the metabolic roles of its microorganisms are highly conserved: enriching the biosynthesis of cofactors and vitamins, in addition to a key role in central carbohydrate metabolism, aromatic amino acids (AA), and ATP synthesis in the lower GI tract (Segata *et al.*, 2011; Human Microbiome Project Consortium, 2012). This has given rise to the notion of a "functional core" of microorganisms rather than a core set of microbial taxa, as the same essential roles can be played by different taxa (Lloyd-Price *et al.*, 2016).

The gut microbiota is influenced by the environment and affected by diet, medications, age, geographic factors, surgical interventions, and host genetics, particularly genes related to the immune system and metabolism (Yatsunenko *et al.*, 2012; Dabrowska and Witkiewicz, 2016; Goodrich *et al.*, 2016). The gut microbiome suffers drastic changes during the first three years of life (Yatsunenko *et al.*, 2012). After that, diet is one of the main factors that shape the gut microbiota (De Filippo *et al.*, 2010; David *et al.*, 2014), and the microbiome continues to evolve all lifelong (Ottman *et al.*, 2012; Odamaki *et al.*, 2016). Once diet is strongly correlated with cultural habits and is affected by geographic factors, such as availability of nutrients and source of carbohydrates, fibers and fat, one can also consider that culture affects the patterns found in the microbiome (Yatsunenko *et al.*, 2012). To study the microbiome is also to study ecology. From an ecological point of view, maintaining sufficient bacterial diversity and richness is important for gut microbiota functional redundancy, adaptability and to provide a certain tolerance against environmental challenges, resilience (Gill *et al.*, 2006). Western diets, rich in calories and refined sugar, are associated with lower richness in microbial communities at individual level (alpha diversity) and higher variation among individuals (beta diversity) when compared with diets high in fiber and relatively low in calories (Martínez *et al.*, 2015). Individuals who consume a Western type diet with high-energy and high-fat intake present changes in metabolic and immune biomarkers, such as a higher body mass index and higher levels of inflammatory markers than those who follow a high-fiber, low-calorie diet (Cani *et al.*, 2009). Taken together, these facts have led to associations between microbial richness and health. Once microbial richness is strongly associated with diet patterns (De Filippo *et al.*, 2010; Cotillard *et al.*, 2013; Sonnenburg and Sonnenburg 2014), both the composition and energy content of one's diet are important modulators of the microbiota (Oriach *et al.*, 2016). Diet is a crucial driver of the composition of the microbial community from childhood to old age (Kashtanova *et al.*, 2016) and has the potential to alter the bacterial metabolite profile, thus influencing the host's metabolism both directly and indirectly.

The major bacterial metabolites known to influence the host include short-chain fatty acids (SCFAs) and vitamins. SCFAs are organic monocarboxylic acids with six or fewer carbon atoms, generated by anaerobic fermentation of indigestible dietary fibers (such as cellulose, xylans, and inulin) in the gut. The main SCFAs produced as a result of these fermentation processes are butyrate, acetate, and propionate. SCFAs are absorbed by the host and are important energy sources, corresponding to 10% of the energy source in a Western diet. Portal and hepatic veins contain large amounts of SCFAs (Cummings *et al.*, 1987). SCFAs also stimulate growth of bacteria in the genera *Lactobacillus* and *Bifidobacterium*, these playing a key role in colon physiology and metabolism (Roy *et al.*, 2006) and influencing the immune and inflammatory responses (Maslowski and Mackay, 2011; Tremaroli and Bäckhed, 2012; Lopez-Legarrea *et al.*, 2014). *In vitro*, SCFAs increase the production of anti-inflammatory cytokines, such as IL-10, and decrease production of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α (Vinolo *et al.*, 2011). Production of SCFAs also promotes transcription of the *PTH1* gene, which encodes tryptophan hydroxylase, the rate-limiting enzyme of serotonin synthesis in the gut (Reigstad *et al.*, 2015). SCFAs are also generally involved in G-protein signaling, modulation of cell signaling, cell–cell interactions, gene expression, immune function, and neurotransmitter synthesis and release (Nakao *et al.*, 1998; Le Poul *et al.*, 2003; Nguyen *et al.*, 2007; Han *et al.*, 2014; Nankova *et al.*, 2014). Several physiological effects, including regulation of energy homeostasis, obesity, immune system functions, cancer, and cerebral function, as well as histone deacetylase (HDAC) inhibition, have been associated with butyrate (Koh *et al.*, 2016). Specific host transporters and receptors are available for butyrate, and it is also used by colon cells as a source of energy through beta-oxidation (Stilling *et al.*, 2016). Furthermore, acetate and propionate can be used by the liver for lipogenesis and gluconeogenesis, respectively (Janssen and Kersten, 2015). The potential for modulation of host metabolism and genetics by the gut microbiota suggests that the role of this factor warrants closer attention. This is especially true in IEMs in which metabolic pathways are originally altered, as the microbiome may act to reinforce metabolic pathways that are advantageous or disadvantageous to the host, with a direct impact on phenotype.

The evidence for a role of the composition of the human gut microbiota and its metabolites in health and illness becomes increasingly stronger (Sharon *et al.*, 2014; Coleman and Nunes, 2016; Rooks and Garrett, 2016). Changes in the GI tract microbiota induce metabolic changes with systemic effects (Tremaroli and Bäckhed, 2012; Ochoa-Repáraz and Kasper, 2014; Sharon *et al.*, 2014), and current research seeks to characterize microbiota–host interactions to elucidate the depth and breadth of this influence.

Some conditions, such as liver and bowel diseases and *Clostridium difficile* infection, are already being treated with microbiota-modifying therapies. These include probiotics, prebiotics, antibiotics, and fecal transplant (Sheth *et al.*, 2016; Young, 2017). Probiotics are living microorganisms that, when administered at an appropriate concentration, can confer health benefits to the host, while prebiotics are indigestible components of foods that benefit the host by promoting growth or activity of a specific bacterial species or community in the colon. Fecal transplant is the administration of fecal matter from a healthy donor to a diseased individual, with the objective of restoring the typical microbial community of the healthy gut. These strategies can be used jointly or in isolation to restore the balance of the intestinal microbial community in the event of dysbiosis, which is any change to the composition of resident commensal communities relative to the community found in healthy individuals.

Inborn Errors of Metabolism (IEM)

IEMs are individually rare diseases, but as a group they are fairly common. Currently, more than 600 known human diseases are classified as IEMs (Alfadhel *et al.*, 2016). Classically, IEMs are defined as a set of monogenic (single-gene) diseases that cause protein dysfunction, with partial or total loss of enzyme activity; however, IEMs can be pleiotropic, and may involve virtually any organ or system. Clinical onset may occur from even before birth up to adulthood (Sharer, 2011), and environmental triggers may be crucial determinants of individual phenotype (Lanpher *et al.*, 2006). In an individual IEM, one primary metabolite flux is affected. In complex disease, however, a whole network of metabolite fluxes might be subtly altered to contribute to the overall phenotype. This concept of metabolic flux is essential in the translation of genetic and environmental factors into the phenotype or threshold for disease (Lanpher *et al.*, 2006). Even a single metabolite defect can affect several secondary metabolic pathways, with a greater or lesser degree of environmental influence, to contribute to each patient's specific phenotype.

The treatment and management of IEMs are always individualized, based on each patient's diagnosis and phenotype, and there is broad heterogeneity even within each category (Argmann *et al.*, 2016). Despite this heterogeneity in management approaches, the specific treatment usually falls into one of three classes: (I) enzyme replacement therapy, to replenish the deficient enzyme; (II) substrate reduction therapy; or (III) dietary treatment, although organ transplantation is also used in some cases (Ezgu, 2016). Additional non-specific treatment may be necessary, depending on the presence of comorbidities, such as neuropsychiatric disorders in PKU patients (Bilder *et al.*, 2017), or renal and neurologic impairment in patients with tyrosinemia type I (Santra *et al.*, 2008; Chinsky *et al.*, 2017). Given the importance of diet to the microbiome, we will

primarily address dietary therapy in this review, with a secondary focus on the importance of the microbiome in allogeneic hematopoietic stem-cell transplantation (HSCT).

Dietary treatment for IEMs may be employed as monotherapy or adjuvant therapy. Its purpose is to eliminate or reduce whichever toxic compound that accumulates in the body (Schwartz *et al.*, 2008). However, this form of therapy has several limitations, including overload and/or deficiency of certain food groups and nutrients (Crenn and Maillot, 2007; Boyer *et al.*, 2015). Theoretically, diets restricted or excessively rich in certain nutrients may prompt a state of intestinal dysbiosis with systemic effects, leading to malnutrition, obesity (Henao-Mejia *et al.*, 2012), type 1 (Wen *et al.*, 2008) or type 2 diabetes (Larsen *et al.*, 2010), inflammatory bowel disease (Ashton *et al.*, 2017; Geirnaert *et al.*, 2017) and liver disease (Lee and Sokol, 2015), as well as a variety of disorders featuring an inflammatory component, symptoms of autism spectrum disorders (De Angelis *et al.*, 2015), and even cancer (Jacqueline *et al.*, 2017; Xu and Jiang, 2017). Studies seeking to identify the effects of dietary treatment and nutrient supplementation on the microbiome of patients with IEMs are still scarce. A summary of this research will be presented below and in Table 1.

Organ transplantation (mainly liver transplantation and HSCT) is also a treatment option for several IEMs (Sirrs *et al.*, 2013; Boelens *et al.*, 2014). Within this context, the microbiome was recently noted as a key factor in graft-*vs.* -host disease (GVHD). Acute GVHD is characterized by rupture of the intestinal barrier, caused by the conditioning regimen administered before HSCT and by leakage of microbe-associated molecular patterns (MAMPs, also known as pathogen-associated molecular patterns or PAMPs), particularly lipopolysaccharide (LPS). The proinflammatory response mounted against these molecules leads to systemic inflammation. Antibiotic treatment in the perioperative period of allogeneic HSCT has been associated with a higher likelihood of GVHD and lower odds of survival, which suggests a potentially pathogenic role of antibiotics through depletion of gut microbiome diversity. The finding that fecal transplant successfully treats GVHD by reconstituting the microbiota has reinforced this theory (Balmer *et al.*, 2014; Melis *et al.*, 2014; Kakihana *et al.*, 2016; Rashidi *et al.*, 2017; Routy *et al.*, 2017; Spindelboeck *et al.*, 2017). Efforts to characterize the influence of the microbiome in complications resulting from organ transplantation are paving the way for new avenues of treatment. Administration of *Lactobacillus*, for instance, appears to be a promising strategy for treatment of GVHD in allogeneic HSCT recipients, although the mechanism of action has yet to be fully understood (Staffas *et al.*, 2017).

Influence of the microbiome on the major organs affected by IEMs

The features of IEMs are highly heterogeneous; however, the nervous system central (CNS) and liver, due to their high metabolic rate, are particularly susceptible to the effects of any metabolic defect (Sahoo *et al.*, 2012). These organs are also closely related to microbiome activity, and a summary of on this matter can be found in Figure 1.

The microbiome has wide-ranging influence on the CNS, with probable effects on metabolism (Fu *et al.*, 2015; Montagner *et al.*, 2016), coordination (Sampson *et al.*, 2016), mood (Slykerman *et al.*, 2017), behavior (Tillisch *et al.*, 2013), cognition (Steenbergen *et al.*, 2015), temperature control (Chevalier *et al.*, 2015), and sensation (Chiu *et al.*, 2013). This influence may begin before birth, via the maternal microbiome (Rautava *et al.*, 2012), and may be perpetuated throughout life, playing essential roles in the development of the blood–brain barrier (Braniste *et al.*, 2014), maturation of the immune system (Chung *et al.*, 2012), and also myelination of the prefrontal cortex (Hoban *et al.*, 2016). Communication between the microbiome and the CNS is two-way, occurring both through metabolites and toxins produced by the bacterial community on the one hand, and via the immune, metabolic, nervous, and endocrine systems on the other (Powell *et al.*, 2017). Over the years, disruption of the microbiome-brain-gut axis has been associated with various diseases. A breach in system homeostasis may occur at any point along this axis. Stressful situations affecting the brain, for instance, may affect the gut microbiome via the hypothalamic-pituitary-adrenal (HPA) axis, with repercussions for immune cell activity and bowel function (Moloney *et al.*, 2014). Bacterial components, in turn, can stimulate secretion of proinflammatory cytokines from epithelial cells, dendritic cells, and macrophages. Knowingly, several neuropsychiatric disorders, including depression, anxiety, schizophrenia, and autism spectrum disorders, are associated with elevated circulating levels of proinflammatory cytokines (Liu *et al.*, 2015a; Petra *et al.*, 2015). In addition to these pathways, cerebral function can also be modulated by microbial metabolites capable of crossing the blood–brain barrier (Li and Zhou, 2016). Pierre and Pellerin (2005) reported that monocarboxylate transporters (MCTs), which transport lactate, pyruvate, ketone bodies, and other SCFAs, are widely expressed in cerebral tissue, and especially so in the cortex, hippocampus, striatum, and cerebellum (Pierre and Pellerin, 2005). In rats, G protein-coupled receptors (GPCRs) activated by propionic acid (PPA) are also highly expressed in brain tissue (Bonini *et al.*, 1997). Antibiotic therapy, which is commonly used in the treatment of some IEMs, depletes the microbiome and can affect levels of neuromodulatory substances (tryptophan, monoamines, and neuropeptides), thus influencing anxiety and cognition patterns (Desbonnet *et al.*, 2015).

Table 1 - Inborn errors of metabolism addressed in this review, main phenotypic features, and overview of management.

EIM (Substrate accumulated)	Affected protein/gene	Main clinical features	Long-term management	Reference
Phenylketonuria (Phenylalanine)	Phenylalanine-4-hydroxylase (*PAH*)	Neurologic impairments, with physical, cognitive, and behavioral consequences, even in well-controlled PKU	Restriction of dietary phenylalanine. Phe-free medical formula. Sapropterin (BH_4) supplementation in responsive patients. Large neutral amino acids (LNAA)	Regier and Greene, 2000; OMIM #261600
Tyrosinemia type I (Tyrosine, maleylacetoacetate, fumarylacetoacetate, and succinylacetone)	Fumarylacetoacetate hydrolase (*FAH*)	Hepatomegaly, acute liver failure, cirrhosis and hepatocellular carcinoma Episodic paralysis and episodic peripheral neuropathy Renal Fanconi syndrome, renal failure, glomerulosclerosis, nephromegaly, nephrocalcinosis Gastrointestinal bleeding, paralytic ileus Pancreatic islet-cell hypertrophy, splenomegaly Rickets, chronic weakness	Dietary management with reduced intake of phenylalanine and tyrosine; Nitisinone Liver transplantation	Das, 2017; Sniderman et al., 2006; OMIM #276700
Urea cycle disorders (Ammonia)	Carbamoylphosphate synthetase I (*CPS1*); Ornithine transcarbamylase deficiency (*OTC*); Argininosuccinate Synthase 1 (*ASS1*); Argininosuccinate lyase (*ASL*), Arginase-1 (*ARG1*); N-acetylglutamate synthase (*NAGS*); Ornithine transporter (*SLC25A15*); or citrin (*SLC25A13*)	Vomiting, lethargy, and behavioral abnormalities. Neurologic impairments. Seizures in acute hyperammonemia. Liver impairments	Dietary management with reduced intake of proteins, Essential amino acids supplementation. Vitamin and mineral supplementation, Medications to increase the nitrogen excretion. Liver transplantation,	Ah Mew et al., 2003; Häberle et al., 2012.
Alkaptonuria (Homogentisic acid and its oxidation products)	Homogentisate 1, 2-dioxygenase (*HGD*)	Urine that turns dark on standing, alkalinization, black ochronotic pigmentation of cartilage and collagenous tissues, arthritis (especially in the spine). Cardiovascular impairments: Aortic and/or mitral valve calcification, coronary artery calcification, aortic dilatation. Urolithiasis, ochronotic prostate stones (in males)	Nitisinone *	Introne and Gahl, 2003; Mistry et al., 2013; OMIM #203500
Propionic acidemia (Propionic acid)	Propionyl-CoA carboxylase (*PCC*)	Central nervous system impairments: acute encephalopathy, lethargy, axial hypotonia, limb hypertonia, coma, seizure, psychomotor retardation, cerebral atrophy, dystonia, cerebellar hemorrhage (rare), ischemic stroke in the basal ganglia (rare). Decreased appetite, feeding difficulties, vomiting, dehydration. Hepatomegaly, pancreatitis. Pancytopenia, neutropenia, anemia, thrombocytopenia. Cardiomyopathy, tachypnea, apnea. Osteoporosis, dermatitis acidemica	L-carnitine, Antibiotics, Low-protein diet, Vitamin and mineral supplementation, Precursor-free amino acid and/or isoleucine/valine supplementation.	Baumgartner et al., 2014; OMIM #606054https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4180313/
Methylmalonic Acidemia (Methylmalonic acid)	Methylmalonyl-CoA mutase (*MUT*)	Central nervous system impairments: lethargy, hypotonia, developmental delay, coma, severe involvement of globus pallidus, delay in myelination, cerebellar hemorrhage (rare), ischemic stroke in the basal ganglia (rare).} Leukopenia, thrombocytopenia. Cardiomyopathy, hepatomegaly, pancreatitis, recurrent episodes of vomiting, interstitial nephritis, chronic renal failure	Same as in PA, plus vitamin B12 in responsive patients.	Baumgartner et al., 2014; OMIM #251000

Table 1 - cont.

EIM (Substrate accumulated)	Affected protein/gene	Main clinical features	Long-term management	Reference
Hemochromatosis type 1 (Iron)	HFE protein, Hemochromatosis gene (*HFE1*)	Heart involvement: cardiomyopathy, congestive heart failure, arrhythmia, cardiomegaly. Liver involvement: cirrhosis, hepatomegaly, hepatocellular carcinoma. Diabetes mellitus. Arthritis. Hypogonadotropic hypogonadism. The severe effects of the disease usually do not appear until after decades of progressive iron loading	Periodic phlebotomy	Seckington and Powell, 2000; OMIM #235200
Trimethylaminuria (Amino-trimethylamine)	Flavin-containing monooxygenase 3 (*FMO3*)	Behavioral/psychiatric manifestations: depression, suicidal, psychosocial problems in school. In some patients: anemia, neutropenia, pulmonary infections; tachycardia and severe hypertension after eating cheese.	Dietary restriction of: Trimethylamine and its precursors including choline and lecithin Trimethylamine N-oxide; Inhibitors of FMO3 enzyme activity, such as indoles. Use of: acid soaps and body lotions, activated charcoal and copper chlorophyllin, antibiotics, riboflavin supplements.	Phillips and Shephard, 2007; OMIM #602079

*Under investigation

As evidence mounts for a systemic effect of the gut microbiome on the host, the liver has also been found to be affected by changes in the microbiome. In addition to its central role in intermediary metabolism (for instance, many enzymes affected by IEM are only expressed in liver) and bile secretion, the liver is the target organ of therapies for metabolic disorders (Brunetti-Pierri and Lee, 2005) and can also be considered a secondary lymphoid organ (Macpherson *et al.*, 2016). Changes in liver physiology are probably caused primarily by DNA methylation processes, covalent histone modifications, and regulation of gene expression by non-coding RNA (*nc*RNA) (Macpherson *et al.*, 2016). In addition to SCFAs, isothiocyanates and polyphenols are also produced by the microbiome, and all of these compounds have the potential to cause epigenetic changes. As the liver receives blood from the gut through the portal vein, it is susceptible to exposure to microbial byproducts that cross the intestinal barrier. In humans and non-human animals alike, whenever liver or bowel disease causes dysfunction of the barrier role played by these organs, there is a breakdown in mutualism between the host and the microbiome, which leads to systemic exposure to gut bacteria and increased immune activation (Chassaing *et al.*, 2015). In these situations, the liver becomes a primary immune barrier that mediates host–microbiome mutualism (Balmer *et al.*, 2014).

Hepatocytes are sensitive to microbial byproducts, and may trigger an inflammatory immune response with systemic effects: even exposure to low levels of LPS induces IFN-γ overexpression and IL-10 underexpression in the liver in animal models of obesity, thus predisposing to the development of steatohepatitis (Yang *et al.*, 1997). On the other hand, deletion of the flagellin receptor TLR5 in mouse hepatocytes has been shown to predispose to hepatic steatosis and fibrosis, as well as other features of the metabolic syndrome. In this study, antibiotic treatment was able to reverse steatosis and related aspects in TLR5 knockout mice, suggesting that mechanisms for clearance of microorganisms capable of gut–liver translocation is essential for maintenance of host systemic health, preventing the chronic inflammation induced by microbial pathogens (Etienne-Mesmin *et al.*, 2016). Taking into account the important immune role of the liver, it makes sense that most patients with cirrhosis and severe liver failure die of sepsis, not of metabolic derangements (Leber *et al.*, 2009), as many of these infections are caused by oral commensals or gut microbiota (Gustot *et al.*, 2009). The dysbiosis state itself impulses inflammatory response and has potential for causing disease. The role of the microbiome in liver disorders is further supported by the efficiency of treating these conditions with probiotics, prebiotics, and antibiotics. Studying the microbiome, hence, may provide a better understanding of complex diseases and lay the groundwork for new therapies (Tilg *et al.*, 2016).

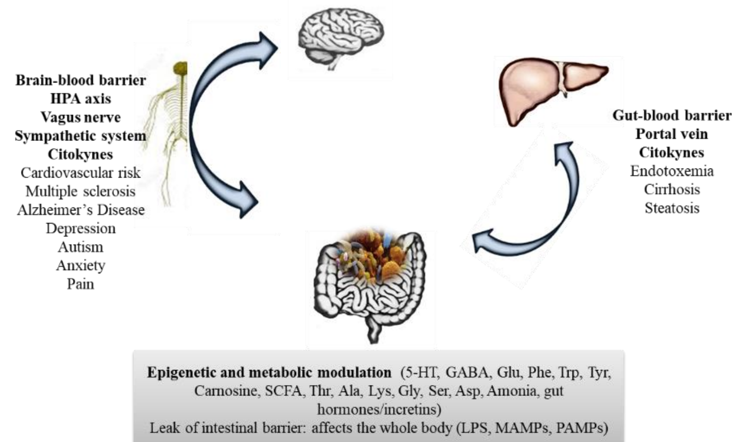

Figure 1 - Known effects of the gut microbiome on the main organs affected in an IEM. In bold are the ways by which the interactions occur. Below are the features related to the gut microbiota and the organs. The gut microbiome produces several metabolites and actively participates in the biosynthesis of vitamins and cofactors, metabolism of carbohydrates, proteins and lipids. The gut microbiota interacts with the whole body via the immune and endocrine systems. The two major organs affected in an IEM are the brain and the liver. In addition to the components of the immune and endocrine systems, the described gut-brain interactions also involve the brain-blood barrier, HPA axis, vagus nerve and the sympathetic system. This may predispose to several diseases, such as increased cardiovascular risk, multiple sclerosis, Alzheimer's disease, depression, autism, anxiety, and also can be related to pain. Interactions with the liver can occur via the portal vein, the gut-blood barrier, and can be involved in several hepatic diseases, most of them linked to endotoxemia.

The microbiome and IEMs: the state of the art

The gut microbiome plays roles in amino acid and carbohydrate metabolism, vitamin and cofactor biosynthesis, and production of SCFAs, in addition to influencing the physiology of the liver, brain, and GI tract, all of which are affected by IEMs. In light of the many important activities of this virtual metabolic organ and its vast impact on the host, some studies have considered the microbiome as a factor that interferes with organic homeostasis in the context of IEMs, and have sought to characterize possible interactions, both endogenous (genetic defect) and exogenous (treatment/diet), with host metabolic pathways, as well as the probable consequences of the presence or absence of specific bacteria and their metabolites on the human body.

Studies of the association between microbiome and IEMs have focused on aminoacidopathies (such as PKU, tyrosinemia, and alkaptonuria), organic acidemias (methylmalonic acidemia and propionic acidemia), and hemochromatosis. The main characteristics of the IEMs addressed in these studies, including their long-term management, are summarized in Table 2. Some possible effects of treatments of IEM on microbiome are showed in Figure 2.

The majority of studies on microbiome–IEM interactions has focused on PKU. One of the most thorough among such studies compared the microbiome of eight patients with PKU to that of 10 healthy individuals by analysis of the 16S rRNA gene. In this study, Pinheiro de Oliveira *et al.* (2016) demonstrated reduced abundance of bacteria in the families Clostridiaceae, Erysipelotrichaceae, and Lachnospiraceae, class Clostridiales, and genera *Coprococcus*, *Dorea*, *Lachnospira*, *Odoribacter*, *Ruminococcus,* and *Veillonella* in patients with PKU, as well as an increase in *Prevotella*, *Akkermansia*, and Peptostreptococcaceae populations. Their metabolic prediction was associated both with starch and glucose metabolism and with AA metabolism (Pinheiro de Oliveira *et al.*, 2016). The authors raised the hypothesis that bacterial enrichment related to LPS biosynthesis, as observed in patients with PKU, might be associated with peripheral inflammation, as indicated by the proinflammatory circulating cytokine profile of these patients (Coakley *et al.*, 2014). In the same study, the authors found a correlation between microbiotic profile and circulating levels of phenylalanine (Phe), which might indicate a relationship between these patients' microbiome, their treatment response, and their phenotype.

Focusing on the potential impacts of prebiotic treatment in individuals with PKU, a study reported by MacDonald *et al.* (2011) analyzed the effects of prebiotic oligosaccharides (scGOS/lcFOS) as an adjunct to the metabolic formula that forms the mainstay of PKU management. As breastfeeding is highly restricted in children with PKU, the authors theorized that a lack of the oligosaccharides present in breast milk might be associated with in-

Table 2 - Summary of experimental studies addressing the role of the microbiome in inborn errors of metabolism.

Reference	EIM	Model	Experimental design	Aims	Findings
Ney et al., 2017	Phenylketonuria	Human	Randomized, controlled, crossover trial; Early-treated PKU subjects consumed, for 3-wk. each, their usual low-Phe diet combined with AA-Formula or GMP; Metabolomics analysis of a subset of plasma and 24-h urine samples; Dietary intake.	To assess metabolites and neurotransmitters derived from Tyr and Trp in plasma and urine samples from subjects with PKU consuming both AA-formula and GMP	Plasma metabolome: 7 of the 40 microbiome-associated compounds showed differential levels with AA-formula compared with GMP; Significant differences in the plasma profile of secondary bile acids; Associated compounds showed differential levels with AA-Formula compared with GMP; Urine metabolome: 7 of 45 microbiome; Individuals fed with AA formula had a 50% higher intake of Tyr and Trp; Differential degradation level of Tyr by intestinal microbes of individuals fed with AA-formula, potential harmful metabolites formed; Higher metabolism of Trp via the kynurenine pathway might be linked with inflammation patterns; Reinforces prebiotic properties of GMP.
Pinheiro de Oliveira et al., 2016	Phenylketonuria	Human	Observational, cross-sectional study, convenience sampling strategy; Review of medical records for Plasma Phe and Tyr levels, and daily Phe intake; Questionnaire including questions on comorbidities, use of medicines, and dietary intake; V4-16S rRNA gene sequencing; Metagenome prediction.	To characterize the microbiome of PKU patients	Decreased levels of Families *Clostridiaceae*, *Erysipelotrichaceae*, and *Lachnospiraceae*, class *Clostridiales*, genera *Coprococcus*, *Dorea*, *Lachnospira*, *Odoribacter*, *Ruminococcus*, and *Veillonella*.
MacDonald et al., 2011	Phenylketonuria	Human	8-week open-label, single-arm, pilot intervention; Infants aged between 4 weeks and 6 months; Formula Phe-free with prebiotic to replace a regular infant formula phe-free without prebiotics; Measurement of Phe levels in blood; Record of stool frequency, size, appearance, and consistency; Stool samples analyzed for pH and bacterial groups (Fluorescence in situ hybridization technique).	Influence of prebiotic scGOS/lcFOS addition to an infant Phe-free protein substitute	Bifidobacteria and lactobacilli–enterococci levels were similar to those of healthy breast-fed infants and greater than those reported for infants on infant formula without prebiotics.
Sawin et al., 2015	Phenylketonuria	Mice C57BL6/J PKU (Pah^{enu2})	PKU (Pah^{enu2}) and wild-type mice were fed with isoenergetic (Aminoacid, GMP, or casein) diets for 8 week; Three experiments were done; Measurement of SCFA by gas chromatography; Quantification of plasma cytokines; Analysis of splenocyte T cell populations by flow cytometry.	Prebiotic effects of GMP	Increased SCFAs levels; Decreased levels of inflammatory cytokines Decreased quantity of the Proteobacteria, genus *Desulfovibrio*.
Durrer et al., 2017	Phenylketonuria	Mice C57BL6/J (PAH^{enu2} mutant)	*In vitro* and *in vivo* test of a probiotic expressing the phenylalanine lyase gene; Measurement of phe plasma pre and post-treatment; Measurement of enzyme activity; Prebiotic mixed into chow; Fecal culture and immunogenic evaluation.	Assessment of a genetically engineered probiotic (GMO)	Reduction of plasma Phe levels in the mouse model of PKU; Survival of GMO Lactobacillus reuteri 100-23C in the mouse gastrointestinal tract, but no permanent colonization; No immune response to transgenic protein.

Table 2 (cont).

Reference	EIM	Model	Experimental design	Aims	Findings
Gertsman et al., 2015	Alkaptonuria Tyrosinemia	Human	Collection of samples from patients with alkaptonuria before and after treatment with NTBC plus samples of Tyrosinemia types I, I and transient patients; Analysis of the sera y tQ-TOF LC/ MS metabolomic platform; Untargeted metabolomics strategy; *In vitro* experiments with cultures of human cells and intestinal flora cultures to identify the nature of the link between 4-HPP and the elevated indoles.	Evaluate the metabolic effects of nitisinone	Increased levels of I3CHO, in patients treated with nitinisone.
Frye et al., 2016	Propionic acidemia (PA)	Human lymphoblastoid cell lines (LCLs)	Measurement of mitochondrial function in ASD and sex-age-matched control LCLs; Incubation with PPA and reactive oxygen species.	Effects of PPA in an unfavorable redox microenvironment	PPA can have both beneficial and toxic effects on mitochondrial function, depending on concentration, exposure duration, and microenvironment redox state.
Buhnik-Rosenblau et al., 2012	Hemochromatosis type 1	Mouse	Comparison between wild-type and genetically deficient mouse; Culture followed by tRFLP and 16S rRNA gene sequencing.	Effects of iron metabolism ($Irp2^{-/-}$ and $Hfe^{-/-}$ genes) on microbiome	$Irp2^{-/-}$ Increased levels of *L. intestinalis* compared to $Hfe^{-/-}$ mice and *L. murinus* compared to both $Hfe^{-/-}$ and WT mice; $Hfe^{-/-}$ Increased levels of *Enterococcus faecium*; Increased levels of *L. johnsonii* to both $Hfe^{-/-}$ and $Irp2^{-/-}$ mice compared to WT.

PKU: phenylketonúria; Phe: phenylananine; Tyr: tyrosine; Trp: tryptophan; scGOS/lcFOS: neutral short chain galactooligosaccharides and long chain fructooligosaccharides; AA: aminoacids; GMP: glycromacropepide; SCFAs: short chain fatty Acids; GMO: genetically modified organism; I3CHO: indole-3-carboxaldehyde (exclusively produced by microbiota); PA: propionic academia; LCLs: Human lymphoblastoid cell lines; PPA: propionic acid; Irp2: iron regulatory protein 2 gene; Hfe: hemochromatosis protein gene. *The identified compounds are either exclusively synthesized or contributed by intestinal bacteria, as well as by human metabolism.

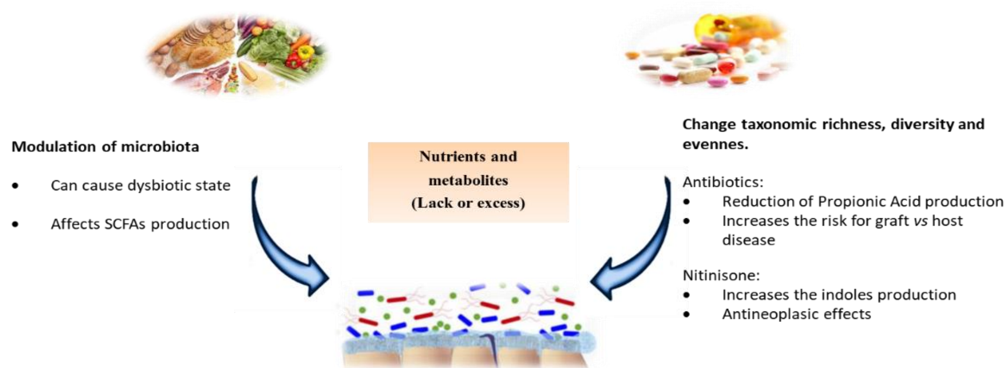

Figure 3. Common treatments used in IEM and its effects over the microbiome. Diet is an important modulator of microbiome and also is a very common treatment for several inborn errors of metabolism. Diets with restriction or abundance of certain nutrients can cause a dysbiotic state leading to an abnormal immune signaling (inflammation), leaking of gut-blood barrier and breaking of the energetic balance of cells, with potential to affect the whole body. Antibiotics, on the other hand, cause rapid and significant drops in taxonomic richness, diversity and evenness. This can bring benefits, as in the case of autism or propionic/methylmalonic acidemia patients, by decreasing the levels of propionic/methylmalonic acid or not, as in the case of organ transplant, once patients treated with antibiotics during perioperative had an increased risk for graft *vs* host disease. The organ transplant is a treatment for several inborn errors of metabolism. Other medicines used for treating this class of genetic disease also can affect the microbiome or metabolite production, like NTBC, that raises the levels of indoles which in turn have antineoplasic effects.

creased fecal pH and reduced bifidobacterial populations, thus predisposing the patient to infections. Administration of probiotics might mitigate this problem. The experiment assessed the dominant bacterial groups and found that the administered prebiotic oligosaccharides were able to maintain bifidobacteria levels and low fecal pH, without altering circulating levels of Phe. Despite the small sample size and lack of statistical power, these findings suggest that supplementing metabolic formula with prebiotics might be an interesting strategy in PKU, as the levels of Bifidobacteria and Lactobacilli–Enterococci at the end of the study were similar to those found in healthy children and higher than those reported in children who took the formula alone, without prebiotics. In the only patient who was previously receiving a diet without prebiotics, there was also a reduction in pathogens such as *C. perfringens* and *C. difficile* (group *Clostridium histolyticum/lituseburense*), *E. coli*, *Shigella*, *Salmonella*, and *Klebsiella* (subgroup Enterobacteriaceae) (MacDonald *et al.*, 2011).

Also regarding prebiotics, recent years have been promising in terms of the use of glycomacropeptide (GMP) as a substitute for Phe-free AA formula in patients with PKU. GMP is highly glycosylated and, when pure, constitutes a natural protein source that lacks the AAs (Phe, tyrosine (Tyr), tryptophan (Trp), histidine, cysteine, arginine) involved in some IEMs, including PKU (Neelima *et al.*, 2013). For now, human trials are seeking to ascertain the efficiency of GMP as a partial (50% formula, 50% GMP) or total replacement for the Phe-free AA formula. In trials, the use of GMP had no significant impact on circulating Phe levels and was preferred by patients over the formula, as GMP is more palatable and, according to patients, provides greater satiety than a formula-based diet alone (Ney *et al.*, 2016; Zaki *et al.*, 2016). This could make GMP an option to increase treatment adherence.

When the urine and plasma metabolome of the individuals with PKU were compared within the groups fed with AA-formula or GMP, differences were found between the metabolite profile linked to the microbes. There were no differences between fasting plasma concentrations of the Tyr and Trp, but individuals fed with AA formula had a 50% higher intake of Tyr and Trp. This can be explained as a result of higher degradation by the intestinal microbes, raising the levels of microbiome-derived compounds from Tyr. Some of these compounds are potentially harmful. There was no differential degradation of Trp, but the metabolism of Trp via the kynurenine pathway was evidenced by higher levels of metabolites linked to this pathway and might be linked with inflammation patterns. Change in plasma profile of secondary bile acids, but not primary bile acids, supports the statement that there are alterations in the gut microbiome with ingestion of AA-formula and GMP, and reinforces the prebiotic proprieties of the GMP (Ney *et al.*, 2017).

Although the effect of GMP on the human gut microbiome has yet to be studied, in mice, GMP was associated with control of Th2-type immune responses, increased *Lactobacillus* and *Bifidobacterium* populations in as little as three days after treatment (Jiménez *et al.*, 2016), elevated levels of SCFAs and reduced levels of proinflammatory cytokines, and reduced Proteobacteria counts (genus *Desulfovibrio*) without affecting circulating Phe levels (Sawin *et al.*, 2015). The genus *Desulfovibrio* is associated with production of hydrogen sulfate, a cytotoxic compound

found at higher levels in patients with ulcerative colitis (Rowan *et al.*, 2010).

Regarding disorders of tyrosine metabolism, Gertsman *et al.* (2015) described the metabolic effect of nitisinone (NTBC or 2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione) in patients with alkaptonuria. Analysis of their metabolic profile showed that indole levels were increased in treated patients as compared with controls. Indoles play a key role in signaling pathways (as building blocks for melanin and serotonin) and intercellular communication, facilitate quorum sensing, and have been uniquely associated with dietary intake and microbial metabolism of tryptophan. Among the indoles found to be increased, indole-3-carboxaldehyde (I3CHO) is produced exclusively by the microbiota, while the other two are produced by human cells (Gertsman *et al.*, 2015). The authors stressed that the reduced form of I3CHO, indole-3-carbinol, a compound also found in cruciferous vegetables, is associated with the prevention of several neoplasms.

Animal experiments also suggest that genetic defects in the host may alter the composition of the gut microbiota, leading to dysbiosis due to a buildup of substances in the cells or lumen of the bowel (Buhnik-Rosenblau *et al.*, 2012). This effect has been observed in hemochromatosis. Hemochromatosis is a disease caused by excess iron absorption by gut cells, which leads to iron overload. This usually becomes clinically detectable in adulthood and is damaging to many organs, including the liver, pancreas (causing diabetes), heart, and skin (Babitt and Lin, 2011). Mutations in the *HFE* gene account for the majority of cases of hereditary hemochromatosis, especially in individuals of Northern European descent (Barton, 2013). In a study of mice with mutations in two genes that encode proteins involved in regulation of iron homeostasis (*HFE*$^{-/-}$ and *Irp2*$^{-/-}$), Buhnik-Rosenblau *et al.* (2012) found abnormalities particularly in resident populations of lactic-acid bacteria, both in *Irp2*-mutant and in *HFE*-mutant mice as compared to controls.

The gut microbiome produces several metabolites, including PPA, a SCFA implicated in several diseases. In autistic populations, the level of the phylum *Firmicutes* is increased and was largely attributable to *Clostridia* class with *Ruminococcaceae* and *Lachnospiraceae* families. The differences in Clostridia species in children with autism spectrum disorder include greater abundance of *Clostridium* clusters I, II, XI and *C. bolteae* (Finegold *et al.*, 2002; Song *et al.*, 2004; Parracho *et al.*, 2005; Williams *et al.*, 2011; Strati *et al.*, 2017). Several *Ruminococcaceae* and *Lachnospiraceae* are known butyrate producers and may thus influence SCFA levels (Louis *et al.*, 2010). So, the treatment with antibiotics can affect producers of SCFA. Some patients' symptoms improve transiently when antibiotics are administered (Sandler *et al.*, 2000; Shaw 2010). Curiously, a similar effect is seen in patients with propionic acidemia, who can experience the same neurodevelopmental complications seen in autism (Witters *et al.*, 2016). Among the various roles played by PPA, it was recently reported to act as a modulator of mitochondrial function. In a study of autism and control cell lines, the effects of PPA depended not only on the concentration of the acid, but also on the level of reactive oxygen species (ROS) present, as ROS influence mitochondrial ability to use PPA as an energy source. Thus, PPA could have beneficial effects in individuals without mitochondrial dysfunction, and harmful effects in individuals with an unfavorable metabolic status and elevated levels of ROS (Frye *et al.*, 2016). In methylmalonic acidemia, which shares several symptoms and management strategies with propionic acidemia, vitamin B_{12} (cobalamin) is also used as treatment in responsive patients, in addition to antibiotics. This vitamin is synthesized by some gut bacteria, and is also a regulator of microbiome composition and function (Baumgartner *et al.*, 2014; Degnan *et al.*, 2014).

The microbiome can also be considered an exogenous source of tetrahydrobiopterin (BH_4), another important metabolite of gut bacteria. BH_4 is a key cofactor for several regulatory enzymes, as Phenylalanine-4-hydroxylase, which catalyzes the conversion of L-phenylalanine to L-tyrosine. The BH_4 has also been shown to improve working memory and cerebral activation (Christ *et al.*, 2013). In rodents, BH_4 production is age-dependent and is related to the presence of Actinobacteria in the bowel, especially *Adlercreutzia equolifaciens* and *Microbacterium schleiferi*. These same species have been identified in the human gut microbiome (Belik *et al.*, 2017). Very little is known about the determinants of responsiveness to BH_4 therapy and its effects on cerebral activity and cognition, but these effects are known to be multifactorial, as they vary across individuals with the same genotype (Pérez *et al.*, 2005). The discovery that BH_4 is naturally produced by gut microbiota has implications for translational medicine, as this cofactor is used in the treatment of some patients with PKU.

The long-term perspective is that elucidation of the metabolic role of the microbiota and identification of which species play these roles will pave the way for manipulating the microbiome, so that pathways beneficial to the host are stimulated, while those harmful to the host are inhibited. In this line, some authors have raised the hypothesis of using methanogenic bacteria normally present in the human bowel to control metabolites such as trimethylamine (TMA), bypassing the normal route of trimethylamine N-oxide (TMAO) production as an intermediate for CH_4 to an alternative pathway (Brugère *et al.*, 2014). In the liver, deficiency in the pathway of TMA conversion into TMAO leads to trimethylaminuria, an IEM that causes strong body odor, impairing the patients' quality of life and interpersonal relations (Mackay *et al.*, 2011). Diets rich in compounds such as phosphatidylcholine, choline, betaine, and L-carnitine generate TMA via the gut microbiota, which is

then converted in TMAO by the liver. High levels of TMAO are associated with increased risk of cardiovascular disease in the general population (Wang *et al.*, 2011; Koeth *et al.*, 2013; Gregory *et al.*, 2015; Liu *et al.*, 2015b). Making the transition from theory into practice, administration of the probiotic *Lactobacillus reuteri,* engineered to express a phenylalanine lyase gene from the cyanobacteria *Anabaena variabilis*, successfully treated mice with PKU. Blood levels of Phe declined after the fourth day of treatment and remained low throughout the experiment, with no permanent colonization of the gut (Durrer *et al.*, 2017), suggesting potential for modified probiotics in the treatment of IEMs.

The creation of genetically modified probiotics design especially to normalize defective metabolic pathways in the host is only one of the many potential advantages of microbiome research. IEMs are characterized by substantial variability in presentation, and genotype alone cannot explain patients' clinical pictures. The microbiome may contribute significantly to factors such as tolerance to certain nutrients and responsiveness to cofactors (and to treatment itself). Studying the microbiomes of patients with IEMs may provide valuable tools for clinical practice, both advancing our understanding of phenotypes and facilitating the development of new biomarkers and therapies.

Main questions about microbioma and IEM and how to address them

There are some important issues involved in the study of the human microbiome in IEM. First of all, most of the diseases that compound the IEM class are rare, and usually there are subclasses within the same IEM. This is the reason why the studies normally have a small number of participants. Second, the microbiome is mainly influenced by diet, and diet overload or restriction is one of most common treatments for IEM. This is one of reasons that make obtaining an adequate control group very difficult. Third, this class of diseases is derived of a metabolic genetic defect, and defects in a metabolic gene also affect the microbiome. So, if a dysbiotic state is observed in this group of patients will it reflect the genetic or the diet effect? Taken together, all the facts above make it very hard to obtain a homogeneous and statistically valid group of untreated patients and make difficult the comparison pre and post-treatment to verify if the altered microbiome is mainly affected by genetic or diet effects. Additional difficulty is added by the fact that several metabolic diseases, if untreated, can lead to severe impacts through life, so IEM patients should start to be treated as soon as possible.

Despite the difficulties, studying the patterns of the microbiome in groups of treated patients offers the possibility to evaluate the real impact of the genetic defect and diet on the microbiome. Patients need lifelong treatment, and the intragroup study of phenotype, microbiome and diet can be elucidative for some ancient questions that remain unknown. PKU patients, for instance, were studied in light of the microbiome by Pinheiro de Oliveira *et al.* (2016) (see Table 2). Even though not capable of answering the question if alteration comes from diet or genetics, a microbiome alteration correlated with Phe blood levels was observed. This is exciting data, due to the fact that it can help explain why some patients are more tolerant to Phe than others, despite having the same genotypes.

In an IEM, the genetic defect and the diet factors coexist, so the measure of macro- and micronutrients ingested is required. Diet has a strong impact on the microbiome, and in spite of patients having similar lines of treatment all over the world, the source of fibers, carbohydrates and proteins can vary geographically and/or culturally. For this reason, microbiome studies should not combine patients of geographically distinct regions or culture to raise the number of participants. Rather, these studies must be done locally and then, if methodologically possible, make comparisons that take into account the dietetic/cultural/geographic factors.

As detailed above, there are several other factors that can influence and be influenced by the microbiome. Important data as sex, age, body mass index, type of birth delivery, breast feeding (duration and transition to solid food), antibiotic and other drug usage, vitamin supplementation, as well as physical exercise, and other diseases (physical and/or mental) must be collected and also analyzed. All subjects included in studies that aim to characterize the microbiome of certain IEMs should be three years or older to avoid the period of drastic changes in microbiome composition due to the typical change in diet during this period. Given that the microbiome varies according to the stage of life and sex, and certain cultures can also exert some influence, the best way to avoid interference of age and sex is the sex-age-matched strategy.

Another useful strategy is based on experimental studies using animal models. This strategy is very important since animal models have less genetic variation and are maintained in a highly controlled environment (that includes diet and/or a germ-free environment). Also, a high the number of subjects can be easily obtained in such research. This is the better model for initial tests of genetically engineered probiotics and correlations with diseases caused by the genetic defect in the absence or presence of the treatment. This kind of study, besides not being capable of fully reproducing the human reality, can work to generate hypotheses and help to provide better strategies and comprehension of studies done in humans.

With the development of NGS tools, procedures are no longer the main limitation for human microbiome studies. Microbiome data is currently obtained by three different approaches: 1) by 16S rRNA gene partial sequencing, 2) by whole DNA shotgun metagenomic sequencing, or 3) by metatranscriptomics (mRNA-seq), to access the active

gene expression pattern For instance, the 16S rRNA gene sequencing method is largely used and has been the first choice method among researchers. Reasons for choosing this approach include the availability of a comprehensive database and scalability. Moreover, studies based in metatranscriptomics require a better control for sample collection to RNA/metabolites processing. Metagenomics, metatranscriptomics and all other "omics", and the associated bioinformatics techniques are allowing comparative analyses in an unprecedented way. All of these tools allow for testing a recent hypothesis related to the presence of a common set of microbial taxa universally present in healthy individuals (Turnbaugh *et al.*, 2007), also known as microbial core. However large variations in the taxonomic composition observed in the human microbiome rapidly refute such a hypothesis (Bäckhed *et al.*, 2012). Due to the well-known microbial functional redundancy in nature, an alternative hypothesis is the presence of a functional core represented by a set of metabolic functions that are performed by the microbiome within a particular habitat, but are not necessarily provided by the same organisms in different people (Shafquat *et al.*, 2014). Still, studies devoted to better understand how deeply the microbiome can affect an organism with critical metabolic pathways that are naturally altered, are just in the early stages. Multidisciplinary efforts need to be done to aggregate modern techniques of sequencing and identification of metabolites that can lead to the phenotype or drug effect in question. Microbial sequencing alone will not be capable of explaining the phenotype, but is a fundamental tool in the understanding of the process. Additional techniques based on metabolomics analysis and RNA-seq, as well as gathering information about the immune system and SCFA levels can offer fundamental pieces of information in the process.

Conclusions

Studies on the microbiome in IEMs are scarce. The effects of the genetic defect itself and of treatment in IEMs, especially in the long term, have yet to be fully understood. As IEMs are commonly managed through dietary intervention (nutrient overload and/or restriction), dysbiosis is a possibility. This dysbiotic status would alter the patients' already compromised metabolic state even further, inducing or worsening abnormalities in secondary metabolic pathways, and thus contributing to phenotypic manifestations, especially liver and brain involvement. Dysbiosis can be treated with antibiotic therapy, dietary prebiotics, or fecal transplant, alone or in combination. The administration of probiotics engineered to at least partly meet the metabolic needs of the IEM-affected host has practically unexplored therapeutic potential and may constitute an intervention that is simple to administer, yet has a major impact on the patients' lives. Collectively, microbiome research in patients with IEMs can not only contribute significantly to our understanding of the pathophysiology of these diseases and to the development of new biomarkers and therapies, but also help to improve the long-term quality of life in affected patients.

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6.2 CAPÍTULO II: Disbiose em pacientes com glicogenoses hepáticas

Título do artigo: “Hepatic glycogen storage diseases are associated to microbial dysbiosis”

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RESEARCH ARTICLE

Hepatic glycogen storage diseases are associated to microbial dysbiosis

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Abstract

Introduction

The gut microbiome has been related to several features present in Glycogen Storage Diseases (GSD) patients including obesity, inflammatory bowel disease (IBD) and liver disease.

Objectives

The primary objective of this study was to investigate associations between GSD and the gut microbiota.

Methods

Twenty-four GSD patients on treatment with uncooked cornstarch (UCCS), and 16 healthy controls had their faecal microbiota evaluated through 16S rRNA gene sequencing. Patients and controls were ≥3 years of age and not on antibiotics. Faecal pH, calprotectin, mean daily nutrient intake and current medications were recorded and correlated with gut microbiome.

Results

Patients' group presented higher intake of UCCS, higher prevalence of IBD (n = 04/24) and obesity/overweight (n = 18/24) compared to controls (n = 0 and 06/16, respectively). Both groups differed regarding diet (in patients, the calories' source was mainly the UCSS, and the intake of fat, calcium, sodium, and vitamins was lower than in controls), use of angiotensin-converting enzyme inhibitors (patients = 11, controls = 0; p-value = 0.001) multivitamins (patients = 22, controls = 01; p-value = 0.001), and mean faecal pH (patients = 6.23; controls = 7.41; p = 0.001). The GSD microbiome was characterized by low diversity and distinct

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microbial structure. The operational taxonomic unit (OTU) abundance was significantly influenced by faecal pH ($r = 0.77$; $p = 6.8e-09$), total carbohydrate ($r = -0.6$; $p = 4.8e-05$) and sugar ($r = 0.057$; $p = 0.00013$) intakes.

Conclusions

GSD patients presented intestinal dysbiosis, showing low faecal microbial diversity in comparison with healthy controls. Those findings might be due to the disease *per se*, and/or to the different diets, use of UCSS and of medicines, and obesity rate found in patients. Although the main driver of these differences is unknown, this study might help to understand how the nutritional management affects GSD patients.

Introduction

Hepatic Glycogen Storage Diseases (GSD) are genetic disorders caused by deficient activity of one of the enzymes involved in the glycogenolysis pathway. The global incidence is estimated at 1 case per 20,000–43,000 live births. The most common types of GSD are GSD I, GSD III and GSD Ixα [1].

In GSD I, glucose-6-phosphate cannot be dephosphorylated to free glucose. There are two major subtypes of GSDI: Ia (~80%), caused by mutations in the *G6PC* gene, and GSD Ib (~20%), caused by mutations in the *SLC37A4* gene. The proteins produced from *G6PC* (catalytic activity) and *SLC37A4* (transporter) work together [2]. GSD Ia involves glycogenolysis and gluconeogenesis, and the clinical manifestations are increased weight, hepatomegaly, failure to thrive, fasting hypoglycaemia, high lactate, hyperuricemia, nephromegaly and hyperlipidaemia [3]. In addition to the features presented in GSD Ia, GSD Ib also presents with susceptibility to recurrent infections, impaired neutrophil and monocyte function, and inflammatory bowel disease (Crohn's-like IBD) [1].

Mutations in the *AGL* gene cause GSD type III, in which the defective glycogen debranching enzyme blocks glycogenolysis, stopping the conversion of glycogen to glucose-1-phosphate [4]. At the same time, gluconeogenesis is enhanced to help maintain endogenous glucose production. Hepatomegaly in type III GSD generally improves with age, but affected individuals may develop chronic liver disease (cirrhosis) and liver failure later in life [5].

GSD IX is caused by the inability of phosphorylase b kinase (PHKA) to break down the glycogen in liver and/or muscle cells. Type IXα glycogenosis is an X-linked disease caused by mutations in the alpha subunit of *PHKA*. The signs and symptoms typically begin in early childhood, but GSD IX is usually milder than the other types [6].

The treatment for the aforementioned types of GSD involves nutritional adjustments primarily, with the periodic and frequent administration of large amounts of uncooked cornstarch (UCCS) and restriction of simple carbohydrates [7] to maintain normoglycaemia and avoid glycogen storage. Usually, higher and frequent doses of UCCS are prescribed for type Ia patients and lower doses for type IX patients. The dose is adjusted according to weight and metabolic demand [8]. GSD III and IX patients may require a hyperproteic diet with fewer restrictions for simple sugars. Sometimes additional medications may be necessary.

During the last decades, our understanding of the human being has changed. We know now that the eukaryote cells encoded by our genome are not the only component of our body. Symbiont prokaryotic cells inhabiting many cavities of our body provide metabolic functions far beyond the scope of our own physiological capabilities [9]. These cells play an important

role in health and disease states [10]. The gut microbes are the most studied human associated microbial communities and consists of trillions of microbes and millions of functional genes [11]. Healthy humans present a remarkable microbial diversity but with similar functions indicating that different microbial communities are associated with a healthy microbiome [12]. The gut microbiome can be influenced by diet, lifestyle, drugs and genetics of the host [13], and has been related to several features present in GSD patients including obesity, IBD and liver disease [14]. This work aimed to investigate possible associations between GSD and the gut microbiota.

Methods

This study was a cross-sectional, observational convenience sampling study, which included 24 GSD patients (Ia = 15, Ib = 5, III = 1, IXα = 3) and 16 healthy controls. All patients were recruited from the outpatient clinics of the Medical Genetics Service at Hospital de Clínicas de Porto Alegre (MGS-HCPA), Brazil from Jan/2016 to May/2017. As inclusion criteria, the subjects (patients and controls) were $\geq$ 3 years old and not on antibiotics. The GSD patients also were required to: a) have a genetic diagnosis of GSD and b) be on treatment with UCCS. The healthy controls were recruited by invitation as they came to routine appointments at Santa Cecília Basic Health Unit, Porto Alegre, Brazil. All subjects received a kit and printed instructions for stool collection, storage, and transport. They were also provided with printed instructions to record three days of dietary information. Each participant collected their own frozen fecal sample and three-day dietary record and submitted them to an outpatient clinic during their next routine check-up. Upon returning to the clinic, each participant answered a brief questionnaire about personal features including weight and height, eating habits, intestinal habits, medicines of recent and/or continuous usage and lifestyle. The study protocol was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre (HCPA). All participants and/or legal guardians signed an informed consent.

As a routine, GSD patients seen at the MGS-HCPA who are on UCCS therapy also receive a multivitamin prescription. Despite optimum dietary treatment other drugs could also be prescribed, mainly for type I patients, such as allopurinol, to prevent gout and urate nephropathy; angiotensin converting enzyme inhibitors, to slow-down or prevent further deterioration of renal function; citrate, to preventing or ameliorating urolithiasis and nephrocalcinosis, in addition to correcting lactacidaemia; statins to treat hypercholesterolaemia [15]; and mainly for Ib patients, G-CSF to treat neutropenia, neutrophil dysfunction and IBD; and the intestinal anti-inflammatory mesalazine (5-amino-salicylic acid), also to treat IBD [16].

Nutritional assessment, clinical data and statistical analysis

Macro and micronutrients intake by the subjects were estimated from the three-day food records through the Nutribase software (NB16Cloud, CyberSoft, Inc., Phoenix, AZ, USA). The daily nutrient intake of each participant was the sum of the nutrients of each food item. The average of the three-day intake was used for further analysis. Multivitamin consumption and other medications were not included in the nutritional assessment but were considered as variables that potentially were modifying the gut microbial composition, so they were tested by Permutational Multivariate Analysis of Variance. Clinical data, such as IBD and other relevant conditions, were accessed from medical records. BMI-for-age and Z-scores were calculated within the World Health Organization (WHO) AnthroPlus software suite. A qualitative classification for this data followed the WHO criteria [17].

Statistical analysis among the groups was performed using PASW Statistics for Windows software (Vs18.0, 2009, SPSS Inc., Chicago, USA). Numerical variables were

compared using the Mann-Whitney U test. Categorical variables were compared using X^2, Fisher's exact test or Continuity Correction, when necessary (with statistical significant determined by the threshold $p \leq 0.05$). Statistical analyses with the microbiome feature are described below.

Bacterial DNA extraction, *16S rRNA* gene amplifications and sequencing

The bacterial DNA was isolated from 0.3 mg of frozen faecal sample with QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) (Qiagen) according to manufacturer instructions and stored at -20˚C until use. The NanoVue system (GE Healthcare, Chicago, IL, USAGE Healthcare) was used to assess the quality of extractions for downstream applications. For the sequencing step, the library was prepared following the procedures described by Barboza et al. [18]. Briefly, region V4 of 16S rRNA gene was amplified with the barcoded bacterial/archaeal primers 515F and 806R [19] in a reaction containing 2U of Platinum Taq DNA High Fidelity Polymerase (Invitrogen, Carlsbad, CA, USA), 4 μL 10X High Fidelity PCR Buffer, 2 mM MgSO4, 0.2 mM dNTPs, 0.1 μM of both the 806R barcoded primer and the 515F primer, 25μg of Ultrapure BSA (Invitrogen, Carlsbad, CA, USA) and approximately 50 ng of DNA template in a final volume of 25 μL. After an initial denaturation step of 5 min at 95˚C, 30 cycles of 94˚C for 45 s, 56˚C for 45 s and 72˚C for 1 minute were performed, followed by a final extension step of 10 min at 72˚C. After visualization on agarose gel 1.5%, the PCR products were purified with the Agencourt AMPure XP Reagent (Beckman Coulter, Brea, CA, USA) and the final concentration of the PCR product was quantified with the Qubit Fluorometer kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. Finally, the reactions were combined in equimolar concentrations to create a mixture composed of 16S gene amplified fragments of each sample. This composite sample was used for library preparation with the Ion OneTouch 2 System using the Ion PGM Template OT2 400 Kit (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing was performed with Ion PGM Sequencing 400 on the Ion PGM System using Ion 318 Chip v2.

16S profiling data analysis

The Fastq files exported from the Ion PGM System were analysed with the BMP Operating System (BMPOS) [20] according to the recommendations of the Brazilian Microbiome Project [21]. Briefly, an Operational Taxonomic Unit (OTU) table was built using reads truncated at 200 bp and quality filtered with a maximum expected error of 0.5. After removing singletons, the sequences were clustered into OTUs at cutoff of 97% similarity, and chimeras were checked and removed to obtain representative sequences for each microbial phylotype. Taxonomic classification was carried out in QIIME version 1.9.1 [22] based on the UCLUST method against the SILVA ribosomal RNA gene database version v132 [23] with a confidence threshold of 80%. Downstream analyses were carried out with dataset rarefied to the minimum library size [24,25] in the R environment [26] using the phyloseq package [27] and vegan package [28]. The online software Microbiome Analyst [29] was used to further detect microbial biomarkers associated with GSD patients. After Cumulative Sum Scaling (CSS) normalization [30], the dataset was analysed by the non-parametric factorial Kruskal-Wallis (KW) sum-rank test followed by Linear Discriminant Analysis [31]. To make sure the biomarkers observed were not only driven by IBD-like patients, we performed one analysis using the full dataset and another analysis excluding all four IBD-like patients and matched controls.

Faecal calprotectin assay and pH measurement

Frozen faecal samples of patients and controls were thawed and aliquoted at room temperature (20˚C) to perform the pH measures and calprotectin assay. To determine the faecal pH, the samples were diluted 1:10 (w/v) in distilled water. After homogenization and incubation for 5 min at room temperature, the faecal pH was measured by an electronic pH-meter (K39-1014B, KASVI, PR, Brazil) three minutes after complete electrode immersion.

The faecal calprotectin was quantified from 100 mg of faecal sample with the RIDASCREEN Calprotectin test (R-Biopharm AG) according to the manufacturer's instructions. Calprotectin is a calcium-/zinc-binding protein, highly stable and resistant to degradation by intestinal contents (pancreatic secretions, proteases, and bacterial degradation). It is mainly produced by neutrophils in inflammation and has been amply confirmed in intestinal inflammatory diseases [32]. Calprotectin was evaluated to verify gut inflammation across groups and its influence over the number of OTUs. Due to the small sample size of GSD III and IXα, just the subtypes Ia and Ib (groups containing >15% of total sample) were compared. Results for GSD Ia and GSD Ib patients were presented as median (Q1-Q3) and as min-max to GSD III and IXα. To test the correlation among calprotectin and OUT richness, patients who were on mesalazine were excluded from analysis.

Results

The characteristics of the patients and controls are summarized in Table 1. The nutrient intake varied significantly between groups (S1 Table); the largest variation observed was the higher total carbohydrate and calorie intakes in the GSD group due to UCCS usage. The amount of protein consumed (g) and the number of calories derived from proteins did not differ between patients and controls. However, the percentage of total caloric intake from proteins was lower in patients. Patients ingested less fat (g and Kcal/day) and had a lower percentage of fat in the diet. Regarding micronutrients, patients' diet was poor in calcium and sodium, and in vitamins B3, H, D and E in comparison to the control group's diet.

The intakes of macro and micronutrients were similar among all the GSD types, with some kcal variation from carbohydrate intake due the difference in UCCS consumption among groups (S2 Table).

Overall 16S rRNA sequencing results, sequence quality control and control for confounding variables

After quality filtering of the 16S rRNA reads, a total of 1,786,582 high-quality sequences longer than 200 bp were obtained. To analyse whether the number of sequences from each sample was representative of the underlying bacterial community, sequence coverage was calculated (S3 Table). An average of 44,664 sequences per sample was obtained with average sequence coverage of 0.99 at the 3% dissimilarity level. This sequencing depth was sufficient to obtain excellent representation of the microbial community in these samples.

Results for suspected confounding variables that potentially were modifying the gut microbial composition are presented at Table 1 and S1 Table. The gut microbial communities were not affected by sex, age, nor the nutritional status of the subjects tested. Faecal pH was lower in patients (6.23) than in controls (7.41), and this variable affected the presence/absence and abundance of the gut microbes, with a reduced OTU count in lower pH. Only 18% of controls (n = 3) and 41% of patients (n = 10) used antibiotics within the 6 months prior to data collection. The use of antibiotics within the 6 months prior to sampling did not affect the presence/absence of microbes ($p = 0.252$) nor microbial relative abundance ($p = 0.179$) in these samples.

Hepatic GSD is associated with an abnormal gut microbial community

The analysis of overall microbial community structure revealed significant differences between patients and controls (Fig 1). According to the PERMANOVA, the microbial community structure between patients and controls differed by the presence and absence of taxa (r^2 = 0.182; p = 0.003) and by their relative abundances (r^2 = 0.166; p = 0.001). The analysis indicated that the relative abundance of taxa contributed 16% of the variation in the microbial community between patients and controls while the presence/absence of specific taxa contributed 18% to that variation.

Microbial diversity as measured by richness of OTUs and by the Shannon diversity index also differed significantly ($p < 0.01$) between patients and controls (Fig 2). On average, control stool samples possessed 184 OTUs while the patients had only 100 OTUs. The average Shannon diversity index was 3.49 and 2.48 in controls and patients, respectively. Together, these beta and alpha diversity analyses indicated that the GSD gut microbiome is characterized by low diversity and distinct microbial structures.

Defining the main taxa associated with the gut microbiota of patients and controls

Specific microbial phylotypes present within the gut community might drive the main differences observed in GSD patients. To find those microbes, biomarker screening analysis was

Table 1. Sample characterization, analysis of potential confounding variables and their effect on microbial communities.

Variable[1]	Patients (n = 24)	Controls (n = 16)	p-value[1]	Microbial community difference between patients and controls			
				Euclidian Metric		Bray-Curtis Metric	
				R^2	p-value	R^2	p-value
Sex (M/F)	14/10	07/09	0.561	0.02942	0.287	0.02964	0.267
Age (yr)	12 (10–19.75)	12.5 (10–23.25)	0.579	0.02895	0.302	0.02775	0.340
Faecal pH	6.23 (5.42–7.16)	7.41 (7.10–7.98)	**0.001**	0.05938	**0.005**	0.08507	**0.001**
Inflammatory Bowel Disease (yes/no)	04/20	00/16	0.136	0.06746	0.009	0.05152	**0.003**
Abdominal pain complaint (yes/no)	09/15	01/15	**0.032**	0.05590	**0.010**	0.04845	**0.009**
Nutritional status* (Obese or Overweight/Normal)	18/06	06/09[†]	**0.044**	0.05199	**0.004**	0.03423	0.121
UCCS intake (g/day)	309.50 (373.7–245.3)	00	**0.001**	0.03698	0.114	0.05594	**0.001**
Drugs (yes/no):							
-Allopurinol	4/20	0/16	0.136	0.02477	0.436	0.02426	0.517
-Antibiotic usage (last 6 months)	10/14	3/13	0.241	0.03047	0.252	0.03200	0.179
-ACE inhibitor	11/13	0/16	**0.001**	0.03351	0.203	0.03919	0.054
-Filgrastim (G-CSF)	5/19	0/16	0.071	0.06654	**0.002**	0.05377	**0.008**
-Mesalazine	3/21	0/16	0.262	0.03089	0.290	0.03389	0.109
-Multivitamin	22/2	1/15	**0.001**	0.04034	0.070	0.05545	**0.003**
-Potassium Citrate	3/21	0/16	0.262	0.02248	0.516	0.02407	0.551
-Proton Pump Inhibitors	2/22	0/16	0.508	0.03068	0.318	0.03087	0.173
-Statins	1/23	0/16	1.000	0.03312	0.286	0.02542	0.486

UCCS: uncooked cornstarch; ACE: Angiotensin-converting-enzyme inhibitor (enalapril maleate); G-CSF: G-colony stimulating factor. Significant ($p<0.05$) events are highlighted in bold.

[1] Numeric variables were reported as medians (Q1-Q3). Due to the not-normal distribution, numeric variables were subjected to the Mann-Whitney test. Qualitative variables were reported as absolute frequency and tested by X^2, Fisher's test or Continuity Correction, as appropriate.

[†] Data for one control was missing. Weight and height were measured when subjects delivered the sample. In this case, a relative drove the sample to the hospital, thus we were unable to do so.

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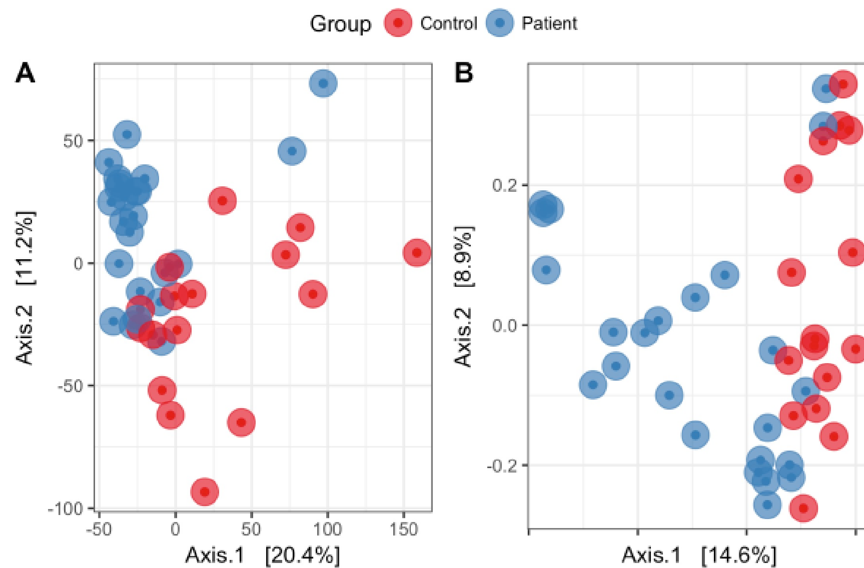

Fig 1. Principal coordinates analysis (PCoA) based on Bray Curtis distance matrix (A) and Euclidean distance matrix (B) show the separation of gut microbiomes between GSD patients and controls. Each point represents a microbial community from one subject; colours indicate the treatment.

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performed at different taxonomic levels. A total of 14 phyla were detected within these samples. However, more than half of the community was dominated by only three phyla: *Bateroidetes* (58% in controls; 47% in patients), *Firmicutes* (34% in controls; 39% in patients) and *Proteobacteria* (5.8% in controls; 10% in patients) (Fig 3). All of the other phyla had very low relative abundances. LEfSe analysis identified three microbial phyla as biomarkers with *Actinobacteria* and *Proteobacteria* overrepresented in patients while *Euryarchaeota* was underrepresented. In particular, *Proteobacteria* presented a very high LDA score (more than 3.9 orders of magnitude), reflecting a marked increase in relative abundance in patients and consistently low abundance in controls. *Firmitutes* had a marginally-significant difference between patients and controls ($p = 0.043$ and LDA score = 4.53 but FDR = 0.07).

At the genus level, nineteen microbial biomarkers were different, both in terms of statistics and biological consistency, between patients and controls (Table 2). Those genera were higher in controls. In patients, those genera were in low abundance and in some cases totally absent. The lack of those microbes might be reflected in the alpha and beta diversity results as mentioned previously (Figs 1 and 2). Besides, *Lactobacillus* and *Escherichia/Shigella* were found to be dominant in patients with a very high LDA score (4.36 and 3.89, respectively), highlighting the biological importance of those microbes in GSD. To remove any biases caused by patients with IBD-like symptoms (n = 4), all IBD-like patients and their respective controls were removed from the dataset and a new biomarker analysis was performed (Table 2). Similar trends as observed within the full dataset were still present in this reduced dataset. However, the *Lactobacillus* genus, found previously in higher abundance in patients was not observed within the dataset without IBD-like patients. On the other hand, *Escherichia/Shigella* was still found to be more abundant in patients than in controls (LDA score = 3.85).

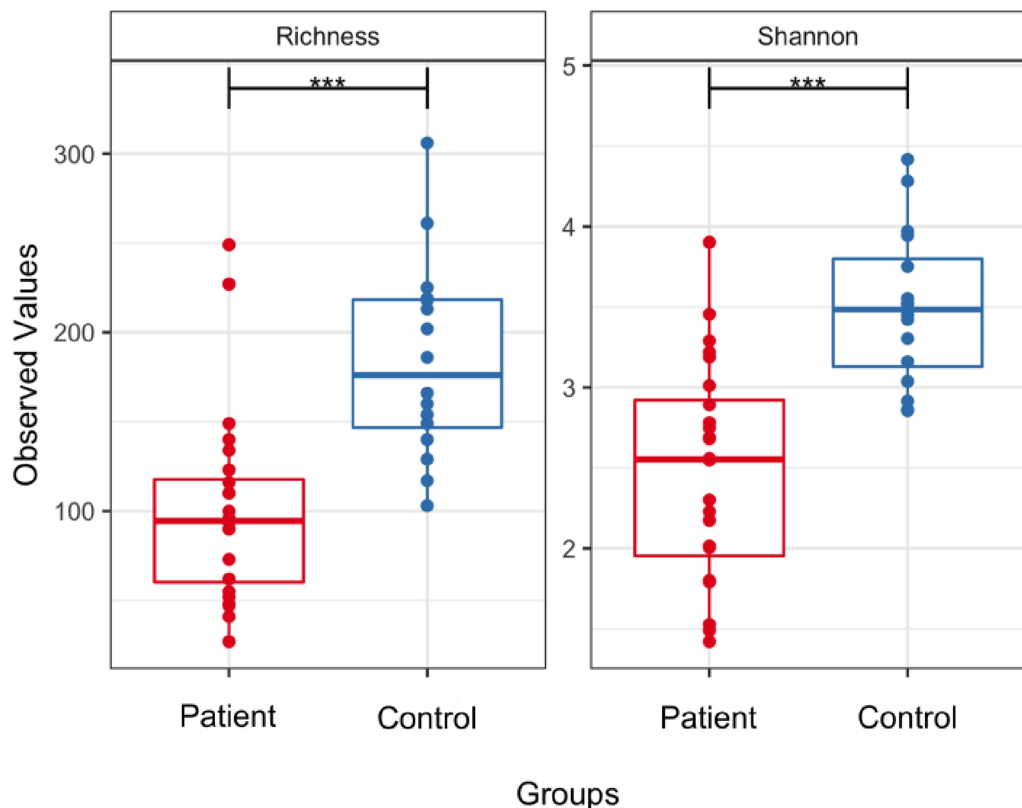

Fig 2. Alpha diversity measurements of microbial communities in the GSD patients and control groups. Each panel represents one alpha diversity measure: Richness = total number of OTUs observed, Shannon = microbial index of diversity. Boxes span the first to third quartiles; the horizontal line within the boxes represents the median. Whiskers extending vertically from the boxes indicate variability outside the upper and lower quartiles. *** indicates a statistical difference between treatments at cutoff $p \leq 0.001$.

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Correlations between the gut microbiota, diet, faecal pH and gut inflammation

Spearman correlations were calculated between the microbiome, diet, faecal pH and calprotectin (Fig 4).

The faecal pH values varied between patients and controls (Table 1), and this was important for shaping their respective differences in gut microbiomes. Differences were determined with the Euclidian distance matrix (for presence/absence of taxa) and the Bray Curtis distance matrix (for relative microbial abundance). Faecal pH was correlated with the total number of microbial OTUs such that higher faecal pH seemed to support more OTUs.

Microbial richness correlated negatively with total carbohydrate but positively with simple carbohydrates (sugar). Calprotectin seemed to have no influence over the microbiome in terms of the number of OTUs (Fig 4). In addition, there was no correlation between this inflammatory marker and gut microbial richness.

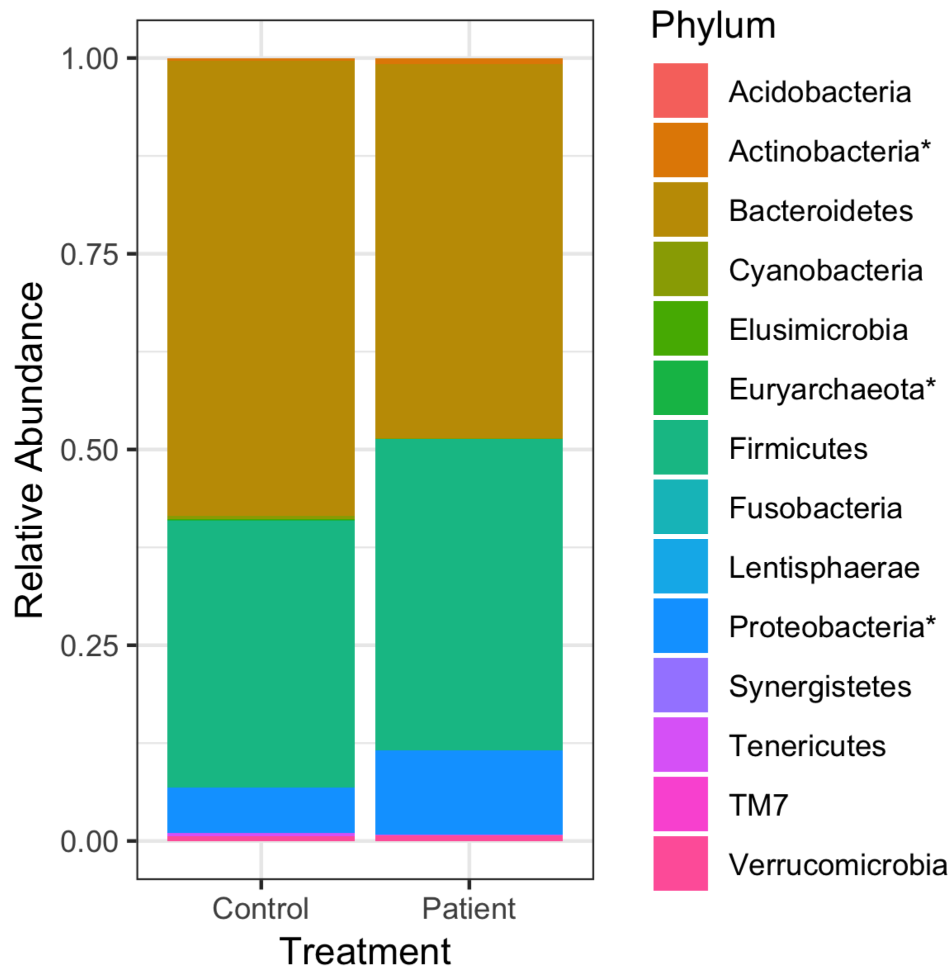

Fig 3. The average relative abundance of phyla found in GSD patients and healthy controls. Phyla followed by an asterisk (*) are different, both in terms of statistics and biological consistency, between patients and controls at p and FDR ≤ 0.05: *Euryarchaeota* (LDA score = 1.75), *Actinobacteria* (LDA score = 3.06) and *Proteobacteria* (LDA score = 3.94). *Firmicutes* was marginally significantly different with $p = 0.064$, LDA score = 4.52 and FDR = 0.112.

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Discussion

This is the first study about the fecal microbiota of GSD patients. In hepatic GSD, high and periodic amounts of UCCS plus dietetic restriction of fast-digestion carbohydrates are the

Table 2. Microbial biomarkers differentiating patients with hepatic glycogenosis diseases and healthy controls.

Microbial genus	Patients	Controls	*p*-values	FDR	LDA score
	Relative abundance (%)				(log 10)
Full dataset	n = 24	n = 16			
Lactobacillus	11.31	0.04	0.009	0.025	4.36
Escherichia/Shigella	6.70	0.96	0.003	0.013	3.89
Alistipes	2.77	9.12	0.005	0.018	-3.22
Subdoligranulum	1.59	1.00	0.012	0.029	2.42
Lachnospiraceae NK4A136 group	1.44	0.89	0.003	0.013	2.48
Faecalibacterium	1.00	3.52	0.016	0.036	-2.98
Ruminococcaceae UCG 002	0.98	3.09	0.001	0.007	-2.79
Bifidobacterium	0.78	0.19	0.004	0.018	3.1
Ruminococcus gnavus group	0.70	0.14	0.007	0.022	3.03
Phascolarctobacterium	0.53	1.31	0.015	0.035	-2.56
Blautia	0.26	0.53	0.002	0.012	-1.55
Odoribacter	0.25	0.53	0.011	0.028	-1.87
Barnesiella	0.22	0.98	0.009	0.025	-2.46
Roseburia	0.18	1.19	0.002	0.011	-2.78
Christensenellaceae R 7 group	0.14	0.80	0.000	0.002	-2.22
Ruminococcaceae UCG 003	0.10	0.60	0.000	0.003	-2.27
Lachnospiraceae UCG 008	0.04	0.26	0.004	0.018	-1.78
Ruminococcaceae UCG 005	0.03	0.25	0.000	0.002	-1.9
Eubacterium hallii group	0.02	0.08	0.000	0.002	-1.39
Anaerostipes	0.01	0.11	0.001	0.009	-1.55
Coprococcus 1	0.01	0.03	0.000	0.005	-0.95
Family XIII AD3011 group	0.01	0.05	0.000	0.002	-1.21
Family XIII UCG 001	0.00	0.03	0.001	0.007	-1.13
Methanobrevibacter	0.00	0.17	0.001	0.007	-1.78
Ruminococcaceae NK4A214 group	0.00	0.08	0.001	0.007	-1.5
Dataset without IBD-like patients[*]	n = 20	n = 14			
Escherichia/Shigella	6.47	0.92	0.003	0.027	3.85
Alistipes	2.97	9.76	0.008	0.039	-3.28
Ruminococcaceae UCG 002	1.12	3.07	0.004	0.028	-1.38
Bifidobacterium	0.81	0.08	0.003	0.027	3.2
Phascolarctobacterium	0.22	1.38	0.004	0.028	-2.74
Christensenellaceae R 7 group	0.17	0.76	0.001	0.016	-2.16
Blautia	0.14	0.39	0.001	0.017	-2.08
Ruminococcaceae UCG 003	0.11	0.61	0.001	0.016	-2.3
Roseburia	0.10	1.15	0.004	0.028	-2.83
Lachnospiraceae UCG 008	0.04	0.19	0.011	0.047	-1.57
Ruminococcaceae UCG 005	0.03	0.20	0.001	0.016	-1.76
Eubacterium hallii group	0.02	0.07	0.000	0.016	-1.32
Anaerostipes	0.01	0.07	0.010	0.047	-1.28
Coprococcus 1	0.01	0.02	0.008	0.039	-0.77
Family XIII AD3011 group	0.01	0.04	0.001	0.017	-1.14
Family XIII UCG 001	0.00	0.03	0.001	0.016	-1.15
Methanobrevibacter	0.00	0.17	0.003	0.027	-1.81

(*Continued*)

Table 2. (Continued)

Microbial genus	Patients	Controls	*p*-values	FDR	LDA score
	Relative abundance (%)				(log 10)
Ruminococcaceae NK4A214 group	0.00	0.08	0.003	0.027	-1.53

[*] Four IBD-like (Inflammatory Bowel Disease) patients and matched controls were excluded from the dataset to make sure the biomarkers observed were not only driven by these patients.

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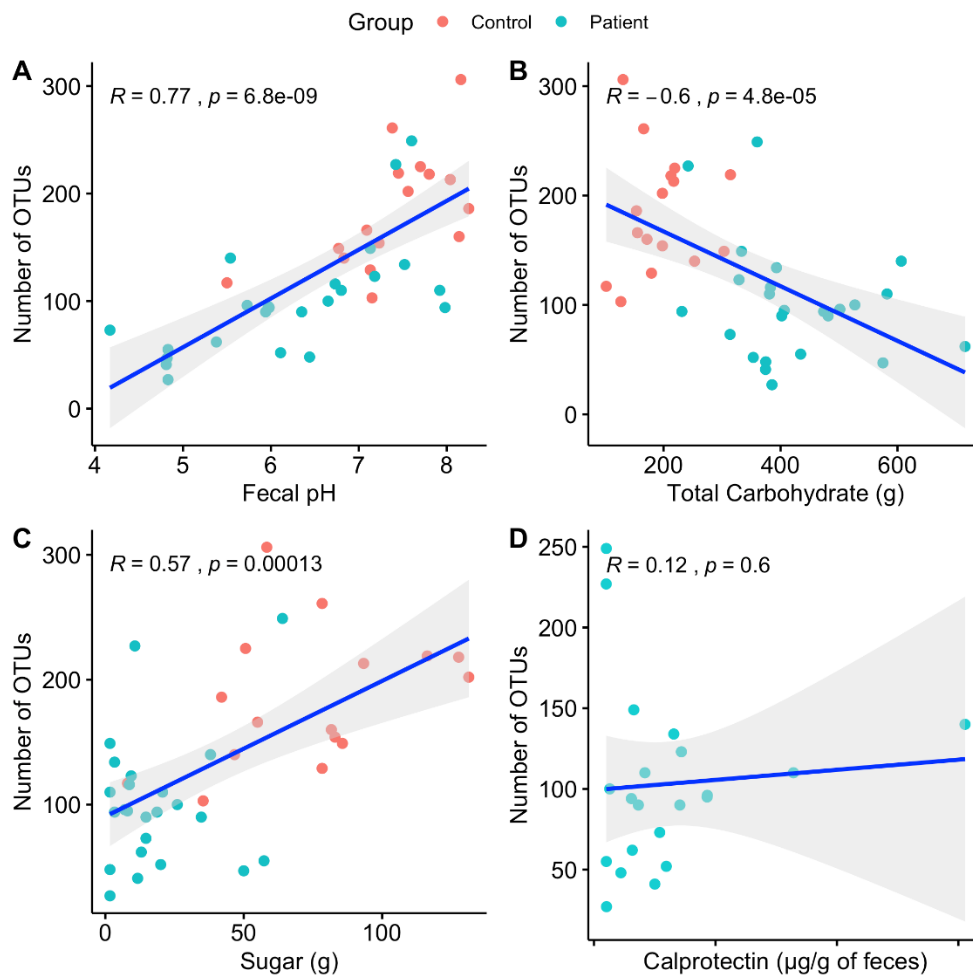

Fig 4. Correlations between the microbiota and diet, faecal pH, and gut inflammation.

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main way to treat the genetic impairment in the glycogenolytic pathway. Our data suggest that the overload of UCCS can lead to low fecal pH by favouring some bacterial genera capable of utilizing complex carbohydrates in detriment of others. The low fecal pH, in turn, also acts as an environmental selection factor to the bacteria in the lumen. Dysbiosis has been associated with IBD and obesity. IBD includes inflammatory bowel diseases of unknown aetiology and has two main forms: ulcerative colitis and Crohn's disease (CD). CD is a chronic disease that can affect any region in the digestive tract but is more likely to involve the small and large intestines and the perianal region [33]. Enteropathy is related to type I patients, and despite GSD Ib patients are classically described as prone to IBD-Crohn's-like due the impaired neutrophil activity, this does not explain why patients with GSD Ia also displayed serologic markers altered for IBD, even if asymptomatic [34]. Its not clear if UCCS is the cause of obesity in GSD patients [35], but the microbiome might be associated with it. Here we discuss why the changes in microbiota could be considered as a factor of influence in the phenotype of these patients and why the UCCS usage, even though not exclusively, is an important factor that contribute to that.

Since the introduction of UCCS treatment for GSD, the focus changed from mortality to morbidity and control of long-term complications [36], such as metabolic syndrome and related symptoms [37,38]. GSD type I patients are prone to obesity, and it is suspected that UCCS contributes to the aforementioned features [35,39]. GSD I patients also are subject of heavier doses of UCCS and more restrict diet in comparison with types III and IX [35]. Regarding antibiotics, although its usage clearly drives changes in the gut microbial community, subjects who were treated with antibiotics within 6 months prior to data collection, but not during the study itself, were not affected by the previous antibiotic usage.

We found that the phyla *Actinobacteria* and *Proteobacteria* were overrepresented in patients while the *Euryarchaeota* was underrepresented. The microbiome of GSD patients present low diversity and was highly dominated by *Escherichia/Shigella*.

One possible driver of the differences in gut microbiomes between patients and controls is UCCS overload, which creates an acidic environment [34,40]. In the human body, acids are generated by regular metabolic activities and through the daily intake of food [41]. Fecal pH was lower in patients than controls and stool acidification might lead to an alteration in the relative abundances of fermenting bacteria, decreasing the conversion of unabsorbable starches to short chain fatty acids (SCFAs) [34].

SCFAs, including butyrate, are compounds made by bacteria in the gut that affect several physiologic functions and serve anti-inflammatory roles [42]. Fecal pH was associated with beta diversity and bacterial families belonging to the Clostridia class, an important producer of butyrate in the gut. Several genera of SCFA-producing bacteria—*Coprococcus*, *Blautia*, *Anaerostipes*, *Odoribacter* and *Faecalibacterium*—were decreased in patients. Those genera were also identified in paediatric patients with Crohn's Disease [43]. Besides, *Coprococcus* and *Faecalibacterium* were found to have significantly low abundance in patients with nonalcoholic fatty liver disease, independently of body mass index and insulin resistance [43].

The bacterial species residing within the mucous layer of the colon may influence whether host cellular homeostasis is maintained or inflammatory mechanisms are triggered. A mutualistic relationship between the colonic microbiota, their metabolic products and the host immune system is likely involved [44]. The phylum *Proteobacteria* was more abundant in patients than in controls while the phylum *Euryarchaeota* was less abundant. *Proteobacteria* is a gram-negative phylum with an outer membrane mainly composed of lipopolysaccharides (LPS), which are known to sustain systemic levels of low-grade inflammation [45]. Higher levels of *Proteobacteria* can be considered a strong marker of dysbiosis [46]. This phylum is prevalent in patients with liver cirrhosis [47]. Several serological markers for IBD were altered in

GSD-Ia patients [34], and GSD Ib patients are prone to IBD CD-like. Despite the fact that calprotectin seemed not to influence the number of OTUs gut inflammation (calprotectin >50μg/g) was verified in several patients. GSD type Ib patients have shown a concentration of calprotectin ≤50μg/g, except for one patient, who had an active IBD diagnosed in the same week. This might be due to a remission state and the use of anti-inflammatory mesalazine by these patients.

In general, dysbiosis can be categorized as a) loss of beneficial organisms, b) excessive growth of potentially harmful organisms and c) loss of overall microbial diversity. These three categories often occur at the same time [48]. Dysbiosis has been implicated in a wide range of diseases, including IBD, liver disease and obesity, that are secondary manifestations in GSD patients [49]. The reason for dysbiosis remains unclear, but the overload of UCCS contributes to those characteristics. The food intake records showed a difference in the intake of calories, mainly due to the administration of UCCS in patients, as well as a difference in microbial signature that is known to be related to obesity. It is not known whether these microbiome changes are a cause or a consequence of the pathophysiologies. However, correcting the dysbiosis can improve health in some patients [50–52]. Dysbiosis can also provide biomarkers for disease detection and management [53].

Conclusion

In this study, we reported significant alterations in the intestinal environments of GSD patients versus healthy controls. Microbiota can be affected by abiotic and biotic factors, namely pH and inflammation, and the differences in these factors between patients and controls might be linked to both genetic disease and UCCS consumption. Several bacterial taxa were different in GSD patients than in controls, and those groups are consistent with the secondary phenotypic manifestations of GSD. The microbiome patterns of these patients may reinforce immune-metabolic pathways that already are altered by genetic impairment, and may also be a factor in the differential individual response to treatment. Patients may gain health and quality of life from the restoration of gut microbial diversity that has been diminished by high UCCS intake. Future research therefore should investigate ways to manipulate the gut microbiome and clarify the possible effects of doing so.

Supporting information

S1 Table. Differences in nutrient mean daily intake between healthy controls and GSD patients and their effect on microbial communities. *Absolute number means that the estimative of ingestion was constant for all the subjects of the group.[1]Mann-Whitney U test. [2]Bray-Curtis. Significant ($p<0.05$) events are highlighted in bold.
(PDF)

S2 Table. Summary of the finding of the GSD patients (n = 24). OTU: operational taxonomic unit; UCCS: uncooked cornstarch; ACE: Angiotensin-converting-enzyme inhibitor (enalapril maleate); G-CSF: G-colony stimulating factor. [1] Numeric variables were reported as Median (Q1-Q3) for GSD Ia and Ib and as Min- Max for GSD III and IXα. Qualitative variables were reported as absolute numbers. P-value was accessed to differences between the groups Ia and Ib. [†]Calprotectin and number of OTUs for patients on and without mesalazine were reported as Min-Max.
(PDF)

S3 Table. Overall description of the 16S rRNA sequencing results among subjects.
(PDF)

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S1 Table. Differences in nutrient mean daily intake between healthy controls and GSD patients and their effect on microbial communities.

Nutrients	Daily intake Median (Q1-Q3)		p-value[1]	Microbial community difference between treatments (r^2; p-value)[2]
	Patients (n=24)	Control (n=16)		
Macronutrients				
Total Calories (kcal total)	2233.83 (1988.16-2867.16)	1520.33 (1143.08-1836.91)	**0.001**	0.045; 0.017
Total Carbohydrate (kcal)	1590.00 (1383.91-2011.00)	747.00 (577.33-827.99)	**0.001**	0.051; 0.006
%Kcal total from carbohydrate	71.66 (67.66-75.25)	49.33 (35.5-55.33)	**0.001**	0.046; 0.018
Total carbohydrate (g)	389.16 (355.00-496.33)	188.66 (154.08-218.58)	**0.001**	0.051; 0.008
* Diet carbohydrate (kcal)	396.55 (240.16-590.53)	747.00 (577.33-827.99)	**0.001**	0.031; 0.159
* Diet carbohydrate (g)	97.33 (58.95-147.50)	188.66(154.08-218.58)	**0.001**	0.033; 0.118
* Uncooked cornstarch (kcal)	1250.33 (970.19-1494.74)	0.00	**0.001**	0.053; 0.004
* Uncooked cornstarch carbohydrate (g)	309.50 (239.58-368.00)	0.00	**0.001**	0.054; 0.002
Total simple sugars (g)	12.33 (4.25-24.66)	78.33 (47.66-91.41)	**0.001**	0.040; 0.051
Fiber (g)	12.33 (10.66-16.91)	13.00 (5.75-20.25)	0.868	0.025; 0.480
Total fat (kcal)	377.66 (260.41-459.41)	544.83 (367.83-581.91)	**0.008**	0.030; 0.204
% Kcal total from fat	14.66 (10.91-19.66)	34.66 (28.78-38.83)	**0.001**	0.049; 0.007
Fat (g)	40.00 (27.83-48.91)	58.16 (38.66-62.41)	**0.007**	0.031; 0.190
Saturated fat (g)	10.33 (7.75-15.41)	22.00 (14.33-24.91)	**0.001**	0.039; 0.049
Monounsaturated fat (g)	13.33 (8.41-19.08)	16.16 (12.83-20.83)	0.269	0.032; 0.174
Polyunsaturated fat (g)	6.16 (3.91-10.00)	7.33 (5.08-10.58)	0.415	0.018; 0.884
Protein (kcal)	299.33 (238.33-339.91)	254.16 (218.16-365.00)	0.307	0.023; 0.609
%Kcal total from protein	13.50 (10.75-14.66)	19.00 (16.08-25.16)	**0.001**	0.033; 0.116
Protein (g)	71.33 (57.25-79.91)	61.16 (52.41-88.33)	0.314	0.023; 0.586

S1 Table. **Differences in nutrient mean daily intake between healthy controls and GSD patients and their effect on microbial communities.**

Nutrients	Daily intake Median (Q1-Q3)		p-value[1]	Microbial community difference between treatments (r^2; p-value)[2]
	Patients (n=24)	Control (n=16)		
Minerals				
Calcium	206.00 (133.66-306.50)	650.16 (382.33-722.83)	**0.001**	0.033; 0.117
Iron	11.00 (8.91-14.41)	9.33 (7.33-15.08)	0.369	0.024; 0.498
Magnesium	175.00 (122.58-210.00)	193.16 (114.74-242.00)	0.619	0.024; 0.529
Manganese	1.50 (1.00-2.00)	1.83 (1.08-2.25)	0.426	0.021; 0.721
Phosphorus	773.66 (633.16-1001.83)	847.66 (617.41-1088.66)	0.649	0.024; 0.489
Potassium	1492.50 (1084.66-1878.24)	1809.66 (1191.08-2155.66)	0.320	0.024; 0.473
Selenium	81.50 (76.16-93.33)	61.83 (49.25-108.50)	0.143	0.033; 0.138
Sodium	1168.33 (651.99-1765.41)	1702.33 (1305.99-2160.74)	**0.034**	0.037; 0.084
Zinc	9.33 (8.00-10.66)	8.16 (6.00-10.75)	0.139	0.030; 0.221
Chromium	0.00	0.00	-	-
Copper	1.00 (1.00-1.25)	1.00 (0.66-1.00)	0.068	0.035; 0.043
Vitamins				
Vit-A (UI)	945.00 (426.50-2758.58)	1297.33 (874.16-5250.16)	0.060	0.021; 0.757
Vit-B1 (mg)	1.00 (0.66-1.00)	1.00 (0.75-1.33)	0.222	0.026; 0.371
Vit-B2 (mg)	1.00 *	1.00 (0.75-1.58)	0.282	0.034; 0.097
Vit-B3 (mg)	16.33 (12.66-19.83)	11.33 (10.00-16.58)	**0.045**	0.025; 0.452
Vit-B5 (mg)	2.83 (2.33-3.66)	3.16 (2.33-4.00)	0.636	0.022; 0.614
Vit-B6 (mg)	1.00 (1.00-1.66)	1.00 (0.41-1.33)	0.173	0.027; 0.328
Total Folate (mcg)	209.83 (159.33-264.83)	243.33 (189.16-373.33)	0.282	0.027; 0.378
Folate, DFE (mcg DFE)	260.33 (190.08-330.00)	281.00 (227.58-434.41)	0.258	0.026; 0.422
Vit-B12 (mcg)	3.33 (2.41-4.50)	3.50 (2.00-4.66)	0.890	0.027; 0.325
Vit-H (mcg)	0.00 (0.00-1.83)	0.00 *	**0.011**	0.037; 0.037
Vit-C (mg)	44.66 (7.50-58.58)	72.83 (26.58-141.33)	0.055	0.027; 0.290
Vit-D (IU)	23.50 (11.83-44.08)	108.66 (51.25-183.08)	**0.001**	0.045; 0.016
Vit-E (IU)	2.33 (1.33-3.58)	3.83 (2.16-7.16)	**0.022**	0.021; 0.716
Vit-K1 (mcg)	39.00 (21.08-54.75)	23.33 (12.75-35.08)	0.090	0.030; 0.187

Significant ($p<0.05$) events are highlighted in bold.

*Absolute number means that the estimative of ingestion was constant for all the subjects of the group.

[1]Mann-Whitney U test

[2]Bray-Curtis

S2 Table. Summary of the finding of the GSD patients (n= 24).

Variables[1]	GSD Ia (n=15)	GSD Ib (n=5)	p-value[1]	GSD III (n=1)	GSD Ixα (n=3)
Sex (M/F)	6/8	3/2	1.00	1/0	3/0[2]
Age (yr)	13 (10-20)	10 (4.0-23.5)	0.405	16	11-29
Faecal pH	5.97(5.38-6.80)	5.98 (4.82-6.58)	0.727	7.98	7.18-7.60
Inflammatory Bowel Disease (yes/no)	0/15	4/1	0.116	0/1	0/3
Abdominal pain complaint (yes/no)	5/10	4/1	0.127	0/1	0/3
Nutritional status (Obese+Overweight/Normal)	12/3	5/0	0.539	1/0	0/3
Calprotectin (μg/g)	108.20(65.58-186.30)	On mezalazine= (45.2-285.5)† No mezalazine= (20.65-44.36)	-	61.58	20.18-143.90
Number of OTUs	166.00 (116-208)	On mezalazine= (51-204)† No mezalazine= (61-85)	0.896	176	290-465
Daily Intake					
-Total Kcal	2315.33 (2103.66-2883.33)	2124.66 (1820.33-2795.00)	0.407	2878.33	1668.66-2400
-Total Kcal/kg	39.30 (34.05-51.44)	58 (33.56-81.01)	0.315	36.43	40.68-63.06
-Total Kcal (from carbohydrate)	1662.33 (1514.33-2129.33)	1565.33 (1219.83-1951.00)	0.239	1900.33	935.66-1368.00
-Kcal from diet	387.92(211.42-626.27)	362.81 (197.39-447.57)	0.694	684.70	380.57-1022.22
-Kcal from UCCS	1300 (1108.44-1766.72)	1153.18 (947.90-1576.52)	0.407	1215.62	345.80-951.43
-Total Carbohydrate (g)	406.33 (374.33-527.00)	382.66 (303.00-480.16)	0.315	473.66	242.00-360.00
-Diet Carbohydrates (g)	94.66 (52.33-155.00)	89.66 (48.91-109.83)	0.694	170.66	94.00-269.00
-UCCS Carbohydrate (g)	321.66 (274.0-431.66)	285.00 (234.00-388.00)	0.359	303.00	91.00-235.00
-Protein (g/day)	71 (55-76.66)	70.33 (55.83-107.33)	0.896	102.33	56.67-101
-Fats (g/day)	30.00 (22.00-47.66)	34 (29.0-48.83)	0.513	54.33	40.67-62.67
Drugs (yes/no)					
-Allopurinol	3/12	1/4	1.000	0/1	0/3
-Antibiotic usage (Last 6 months)	5/10	4/1	0.127	1/0	0/3
-ACE inhibitor	10/5	1/4	0.127	0/1	0/3
-Filgrastim (G-CSF)	0/15	5/0	**0.000**	0/1	0/3

S3 Table. Overall description of the 16S rRNA sequencing results among subjects.

Subject ID	Number of sequences	Coverage	Observed OTUs
		Controls	
C01	46,350	0.999	236
C02	49,504	0.999	231
C03	55,620	0.998	384
C04	44,692	0.999	311
C05	54,335	0.999	246
C06	49,219	0.998	341
C07	41,513	0.998	390
C08	58,964	0.999	274
C09	53,734	0.998	397
C10	40,401	0.998	335
C11	53,303	0.998	419
C12	31,736	0.998	205
C13	27,531	0.996	437
C14	23,019	0.998	229
C15	29,800	0.998	270
C16	17,115	0.994	444
		GSD Patients	
P01	4,724	0.996	51
P02	50,598	0.999	169
P03	44,874	0.999	116
P04	55,299	0.999	166
P05	47,966	0.999	176
P06	20,813	0.997	165
P07	62,846	0.999	85
P08	47,637	0.998	239
P09	39,881	0.999	105
P10	58,674	0.999	138
P11	57,637	0.999	204
P12	59,176	0.999	208
P13	46,914	0.999	175
P14	49,816	0.998	465
P15	68,350	0.999	152
P16	52,118	0.999	61
P17	61,156	0.999	289
P18	58,165	0.999	394
P19	67,259	0.999	290
P20	29,024	0.999	85
P21	37,878	0.999	81
P22	11,018	0.995	155
P23	42,914	0.998	184
P24	35,009	0.998	266

7 DISCUSSÃO

Com a introdução do AMC como alternativa terapêutica eficiente na redução da mortalidade dos pacientes com GSD hepáticas, o foco da atenção volta-se para a qualidade de vida e as complicações tidas como de longo prazo (Moses 2002), tais como o desenvolvimento de sobrepeso e obesidade, síndrome metabólica, doença inflamatória intestinal, adenomas hepáticos e densidade mineral óssea reduzida.

Nosso estudo demonstrou que há disbiose intestinal nestes pacientes e um aumento de bactérias gram-negativas, o que possui implicações no desenvolvimento das comorbidades supracitadas pelo disparo de mecanismos inflamatórios. A obesidade nestes pacientes não é bem compreendida, mas uma das possíveis causas é o desenvolvimento de um ambiente ácido gerado pelo consumo frequente e periódico de AMC (Lawrence et al. 2015), como o verificado em nosso estudo. O pH fecal mais ácido, além de atuar como um modulador ambiental da proliferação bacteriana, uma vez que foi fortemente correlacionado a riqueza de OTUs, pode estar associado a uma queda nos níveis de ácidos graxos de cadeia curta, importantes moduladores da inflamação. Os gêneros e filos bacterianos encontrados alterados estão associados à obesidade, à IBD e a doenças hepáticas como esteato-hepatite não alcoólica e cirrose. O filo *Proteobacteria* foi encontrado aumentado nos pacientes. Este filo é composto por bactérias gram-negativas, as quais contêm LPS na membrana e são indutores de inflamação sistêmica de baixo grau.

O LPS é reconhecido pelo sistema imune humano principalmente pelo TLR4, o qual é capaz de modular os níveis dos transcritos da IL-6 (Nyati et al. 2017). Essa interleucina estava aumentada nos pacientes com GSD-Ib em comparação aos outros tipos, e embora não tenham sido encontradas diferenças estatísticas entre pacientes e controles neste estudo, o valor de p foi limítrofe ($p=0.078$). Apesar da calprotectina não ter demonstrado relação com o pH fecal ou número de OTUs, ela é uma medida de inflamação local, e 75% dos pacientes

com GSD-Ia apresentaram níveis acima do ponto de corte do kit utilizado (>50µg/g) para a detecção da inflamação intestinal. Adicionalmente, a calprotectina é um ativador endógeno de TLR4, estando ligado ao desenvolvimento de processos infecciosos, autoimunes e oncogênicos (Ehrchen et al. 2009).

A quantificação das citocinas nos pacientes com GSD demonstrou que o panorama imunológico é tipo-dependente, ou seja, diferentes tipos de GSD apresentam diferentes níveis de citocinas. Algumas proteínas de importância no processo de reconhecimento de patógenos estavam diminuídas em pacientes, como MCP-1/CCL2 e MDC/CCL22. Além disso, os níveis de triglicerídeos para os pacientes com GSD-Ia sem adenomas foram inversamente correlacionados com IL-10, IL-13, IL-17A e MIP-1β/CCL4. Essas citocinas também estão envolvidas nos processos de inflamação intestinal associados à microbiota (Burrello et al. 2019). Uma vez que triglicerídeos são marcadores de controle metabólico, um metabolismo descompensado parece interferir diretamente no status imunológico desses pacientes.

A fim de integrar os estudos anteriores e embasar futuros projetos de pesquisa, nós encontramos na biologia de sistemas uma alternativa viável para localizar pontos de interação entre microbioma e sistema imune dentro do contexto único dos pacientes com GSD-Ia. As redes de interações primárias foram construídas com proteínas do complexo G6Pase, as duas subunidades da calprotectina e outras duas integrinas previamente descritas como deficitárias na GSD-Ib. Nas redes resultantes da subtração das interações similares encontradas nas redes primárias, a rede que representa as interações proteicas únicas nos pacientes GSD-Ia continha IL-10, TLR4 e IL-6 ligados à única proteína central exclusivamente hepática, indicando que um quadro de disbiose pode ter efeitos mais severos sobre uma rede metabólica já alterada pelo defeito genético. Entretanto, a construção destas redes foi limitada pela disponibilidade de dados "ômicos" no contexto da glicogenose. Isso pode ser verificado pois o nó SLC37A4 e o módulo metabólico associado ficaram ligados por apenas um conector, e

somente se o modo mineração de texto estivesse habilitado. Essa limitação também nos impediu de realizar análise similar para pacientes com GSD-Ib.

Corrigir a disbiose pode melhorar a saúde de alguns pacientes e reduzir os fatores de risco ambientais para as doenças a que estes pacientes já são predispostos por fatores genéticos. Futuros estudos são necessários para compreendermos a extensão da influência do microbioma sobre esses pacientes.

8 CONCLUSÃO

As conclusões desta tese serão apresentadas de acordo com os objetivos específicos enumerados na seção 4.3.

1) Revisar a literatura existe sobre erros inatos do metabolismo e microbiota intestinal.

A literatura existente sobre microbioma intestinal e erros inatos do metabolismo conta com poucos estudos experimentais, os quais são concentrados em fenilcetonúria. Entretanto, os estudos sobre microbioma intestinal e dois dos órgãos mais afetados em um EIM, o fígado e o cérebro, são abundantes. A dieta é um dos principais moduladores da microbiota intestinal, e muitas vezes utilizada como tratamento principal ou adjuvante nos EIM. As dietas para EIM são restritas em alguns nutrientes e/ou sobrecarregadas em outros, havendo um potencial intrínseco do tratamento para o desenvolvimento de disbiose intestinal. A disbiose está relacionada com o desenvolvimento de doenças hepáticas e neurológicas e pode atuar como fator modificador de fenótipo nos EIM.

2) Caracterizar a microbiota intestinal de pacientes com GSD hepáticas em comparação a indivíduos controles, associando-a com dieta, fenótipos e medicamentos utilizados no tratamento.

O microbioma intestinal dos pacientes apresenta uma diversidade e estrutura distinta daquela encontrada nos indivíduos controle, com aumento de Proteobacteria e Firmicutes, diminuição de Bateroidetes, alterações em vários gêneros bacterianos, e enriquecimento de bactérias gram-negativas, como *Escherichia/Shigella*, caracterizando disbiose intestinal. Pacientes e controles diferiram quanto ao consumo de nutrientes, AMC, medicamentos e obesidade. Houve associação significativa entre microbiota, medicamentos, dieta e fenótipos apresentados pelos pacientes. Não foi possível identificar o principal fator que

contribui para a disbiose; entretanto, o pH fecal dos pacientes é mais ácido e fortemente correlacionado com o número de OTUs, provavelmente devido ao uso de AMC. Também houve correlação do consumo de carboidratos totais e açúcar com o número de OTUs. Adicionalmente, os gêneros bacterianos alterados nos pacientes possuem associação descrita na literatura com obesidade e IBD.

3) Caracterizar o perfil de citocinas em pacientes com GSD hepáticas em comparação a indivíduos controle, associando-as a controle metabólico e manifestações clínicas secundárias à doença de base.

Pacientes apresentaram níveis diminuídos de IL-4, MIP-1α, MDC, TNF-β e VEGF em comparação aos controles. Entre os tipos de glicogenoses, pacientes com GSD-Ib apresentaram uma maior variação nas citocinas em ralação aos outros tipos, com maiores níveis de G-CSF do que os outros pacientes, aumento de IL-10 em relação aos pacientes com GSD-Ia, e níveis mais baixos de IP-10/CXCL10 em comparação ao grupo GSD-III/IX. Os níveis de citocinas também apresentaram diferenças quanto a presença de anemia, controle metabólico e adenoma. Verificou-se que os níveis de triglicerídeos possuem correlação com citocinas pró e anti-inflamatórias em pacientes com GSD-Ia.

4) Avaliar possíveis modos de interação entre o microbioma intestinal, citocinas plasmáticas e calprotectina fecal no contexto da GSD-Ia utilizando como ferramenta redes de interação proteína-proteína (IPP).

Detectamos que os pacientes possuem uma abundância maior de Proteobactérias que os indivíduos do grupo controle. Esse filo é composto por bactérias gram-negativas, as quais possuem LPS em sua membrana. O LPS bacteriano é majoritariamente reconhecido pelo TLR4, o qual também é ativado de forma endógena pela calprotectina, e exerce influência sobre os níveis de IL-6 no organismo. O modelo *in silico* utilizado ressalta a importância do TLR4, IL-10 e IL-6 como interatores na rede de proteínas alteradas na GSD-Ia.

9 PERSPECTIVAS

A linha de pesquisa desenvolvida nesta tese continuará sendo explorada numa abordagem integrativa, utilizando dados experimentais e ferramentas *in silico*, tanto na geração de hipóteses como na análise de dados. Para as glicogenoses, estão previstos a quantificação dos principais ácidos graxos de cadeia curta produzidos pela microbiota intestinal e posterior associação com o microbioma e perfil de citocinas. Adicionalmente, pretende-se averiguar se a presença de bactérias gram-negativas nestes pacientes está relacionada com os níveis de citocinas, em especial IL-6 e IL-10, e presença de comorbidades associadas as glicogenoses. A caracterização do microbioma intestinal em outros EIM é de interesse do grupo, de forma que o estudo em pacientes com homocistinúria já está em andamento e planeja-se realiza-lo também nos pacientes com Doença de Gaucher.

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11 APÊNDICES

11.1. Apêndice 1- Carta de aprovação do projeto no Comitê de Ética em Pesquisa)

PARECER CONSUBSTANCIADO DO CEP

Elaborado pela Instituição Coparticipante

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Metagenoma Microbiótico como fator modificador dos Erros Inatos do Metabolismo.

Pesquisador: Ida Vanessa Doederlein Schwartz
Área Temática:
Versão: 1
CAAE: 43110115.6.3001.5347
Instituição Proponente: Hospital de Clínicas de Porto Alegre
Patrocinador Principal: HOSPITAL DE CLINICAS DE PORTO ALEGRE
Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.102.274
Data da Relatoria: 11/06/2015

Apresentação do Projeto:
O projeto "Metagenoma Microbiótico como fator modificador dos Erros Inatos do Metabolismo" é um estudo transversal, controlado, observacional e de base ambulatorial que visa a caracterizar o microbioma intestinal de pacientes com cinco diferentes EIM: Fenilcetonúria, Doença de Gaucher, Glicogenoses Hepáticas, Doença da Urina do Xarope do Bordo e Homocistinúria Clássica. A hipótese a ser testada é a de que existe variação no perfil da microbiota intestinal de cada um dos EIM avaliados, e que a mesma é um fator associado tanto ao tipo e à gravidade das manifestações clínicas, quanto à resposta ao tratamento dos pacientes. A amostra será constituída por 80 casos (Fenilcetonúria= 35; Doença de Gaucher= 20; Glicogenoses Hepáticas= 15; Doença da Urina do Xarope do Bordo= 5; Homocistinúria Clássica= 5) e 160 controles (um controle hígido, pareado por sexo e idade, e um controle familiar (relacionado) ao paciente e que habite a mesma residência).

Objetivo da Pesquisa:
Caracterizar a microbiota intestinal de uma amostra de pacientes com diferentes tipos de EIM (Fenilcetonúria, Doença de Gaucher, Glicogenoses Hepáticas, Doença da Urina do Xarope do Bordo e Homocistinúria Clássica).

Endereço: Av. Paulo Gama, 110 - Sala 317 do Prédio Anexo 1 da Reitoria - Campus Centro
Bairro: Farroupilha **CEP:** 90.040-060
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3308-3738 **Fax:** (51)3308-4085 **E-mail:** etica@propesq.ufrgs.br

Identificar possíveis influências da microbiota intestinal na geração da variabilidade clínica associada aos Erros Inatos do Metabolismo, especialmente em relação a desfechos neurológicos, composição corporal, massa óssea/densidade mineral óssea e taxa metabólica basal.

Avaliação dos Riscos e Benefícios:

Riscos: Os riscos e desconfortos causados pela coleta de fezes são desconforto no momento da coleta. O desconforto associado a esta coleta será minimizado pelo fato de ser realizada no seu próprio domicilio. Possível desconforto para responder as perguntas do questionário e gasto de tempo (cerca de 20 minutos) para responder ao questionário. Risco e desconforto relacionados à coleta de sangue.

Benefícios: Os resultados gerados por esta pesquisa não trazem benefícios diretos para os participantes, mas irão contribuir futuramente para melhorar os tratamentos existentes para os pacientes com Erros Inatos do Metabolismo.

Comentários e Considerações sobre a Pesquisa:

Projeto bem embasado e pertinente do ponto de vista científico.

Projeto aprovado no CEP-HCPA, instituição esta que consta como proponente junto à Plataforma Brasil.

Considerações sobre os Termos de apresentação obrigatória:

O projeto inclui referencial teórico, hipótese, objetivos, justificativa, descrição da amostra e das metodologias, critérios de inclusão e exclusão, descrição do número amostral de conveniência, descrição das análises estatísticas, cronograma de atividades, orçamento e referências bibliográficas. No Formulário da Plataforma Brasil consta a descrição dos riscos e benefícios. Os autores também incluíram o Formulário de Delegação de Funções e os TCLEs para casos e controles.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

O projeto foi originalmente apresentado ao CEP do HCPA, instituição que consta na Plataforma Brasil como proponente e onde ocorrerão as coletas de material e recrutamento dos participantes.

O CEP HCPA emitiu parecer com uma série de recomendações, as quais foram todas atendidas pelos pesquisadores.

O projeto encontra-se em condições de aprovação.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Aprovado.

PORTO ALEGRE, 11 de Junho de 2015

Assinado por:
MARIA DA GRAÇA CORSO DA MOTTA
(Coordenador)

Endereço: Av. Paulo Gama, 110 - Sala 317 do Prédio Anexo 1 da Reitoria - Campus Centro
Bairro: Farroupilha **CEP:** 90.040-060
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3308-3738 **Fax:** (51)3308-4085 **E-mail:** etica@propesq.ufrgs.br

11.2. Apêndice 2- Formulário de coleta de dados e de registro alimentar.

Data da coleta de fezes:

/ /

Data de preenchimento do formulário:

/ /

Identificação

Nº Protocolo:.. Glicogenose tipo: ....

Sexo: ( ) Masculino ( ) Feminino Enzima: ....

Idade: .... Genótipo: ....

Data de nascimento: ..../......... /...........

Dados do nascimento:

Parto: ( ) Normal ( ) Cesárea

( ) A termo ( ) Prematuro, ... semanas

Peso ao nascer: .... g

Comprimento:................................... cm

Apgar 5': ....

Dados Atuais:

Peso atual:... g

Altura atual: .... cm

Intercorrências Gestacionais:

Sangramentos: ( ) Não ( ) Sim Quantos? ....

Ameaça de aborto: ( ) Não ( ) Sim Quantos? ....

Internação: ( ) Não ( ) Sim Quantas vezes? ....

Uso de medicamentos: ( ) Não ( ) Sim

Quais?

....
....
....

Fumou durante a gestação: (   ) Não   (   ) Sim

Consumiu bebida alcóolica durante a gestação: (   ) Não   (   ) Sim

Se sim, qual(is) o(s) tipo(s) de bebida(s) alcoólica(s) consumida(s)?

....

Aleitamento materno (preferencialmente respondido pela mãe)

Amamentou seu filho? (   ) Não   (   ) Sim

Se sim, por quanto tempo?..

Por que deixou de amamentar?..

....

- Aleitamento até 6 meses

Qual o tipo de aleitamento foi oferecido até os 6 meses?

(   ) Exclusivo (só leite materno)

(   ) Predominante (leite materno complementado com chás e água)

(   ) Misto (leite materno, leite artificial, leite de vaca, fórmula, papas, sopas)

Iniciou o leite materno no hospital? (   ) Sim   (   ) Não

Quando amamentou pela primeira vez?

(   ) Durante a primeira hora de vida do bebê

( ) Entre a primeira e a sexta hora de vida do bebê

( ) Após a sexta hora de vida do bebê

Foi dado outro leite a seu filho no hospital? ( ) Sim ( ) Não ( ) Não sei

Qual leite/fórmula foi dado?

Se sim, como foi dado? ( ) No copo ( ) Na seringa ( ) Não sei ( ) Mamadeira

- Aleitamento pós 6 meses

( ) Só complementou com papas ( ) Leite de vaca ( ) Fórmula de segmento

( ) Outro tipo de leite/fórmula. Qual?

....

( ) Papa + FS ( ) Papa + outro leite ( ) Papa + Leite de vaca

( ) FS + outro leite/fórmula ( ) FS + Leite de vaca

( ) Leite de vaca +outro leite/fórmula ( ) Papa+ FS+ leite de vaca

( ) FS+ leite de vaca + outro leite/fórmula ( ) Papa + leite de vaca + outro leite

( ) Papa+ FS+ fórmula de segmento ( ) Papa+ FS+ leite de vaca+ outro leite

Recebeu leite de vaca ou fórmula com lactose até que idade?

....

Que tipo de leite/fórmula/nan utiliza atualmente?..

Medicamentos

Medicamentos atuais:

....

....

....

Utiliza alguma complementação vitamínica? (   ) Não   (   ) Sim

Se sim, quais? ....

....

Por quanto tempo? ....

Algum antibiótico nos últimos 6 meses? (   ) Não   (   ) Sim

Se sim, quais? ....

Usa/usou algum laxante nos últimos 6 meses? (   ) Não   (   ) Sim

Se sim, quais? ....

Informações sobre alimentação atual associada com possíveis alterações na microbiota

Quais destes tipos de alimentos você consome:

(   ) Frutas   (   )Verduras   (   ) Legumes

Com que frequência?

(   ) 1x na semana   (   ) 2 ou 3 x na semana   (   ) Todos os dias   (   ) Quinzenalmente   (   ) Mensalmente   (   ) A cada 3 meses   (   ) Anualmente

Qual o seu tipo de restrição alimentar?

(   ) Sacarose   (   ) Galactose/Lactose   (   ) Frutose (   ) Outra ....

Quantidade de amido por dia (g): .... Frequência: ....

Há quanto tempo segue a dieta: ....

Faz uso de suplementos proteicos? (   ) Não   (   ) Sim

Se sim, qual? ....

Qual a quantidade diária (g)? ....

Ingere alimentos fritos (batata frita, aipim frito, pastéis...) e carnes gordurosas (salsichões e carnes fritas)? (   ) Não   (   ) Sim

Se sim, com que frequência?

(   ) 1x na semana   (   ) 2 ou 3 x na semana   (   ) Todos os dias   (   ) Quinzenalmente   (   ) Mensalmente   (   ) A cada 3 meses   (   ) Anualmente

Utiliza iogurtes ou leites fermentados atualmente? (   ) Não   (   ) Sim

Se sim, com que frequência?

(   ) 1x na semana   (   ) 2 ou 3 x na semana   (   ) Todos os dias   (   ) Quinzenalmente   (   ) Mensalmente

(   ) A cada 3 meses   (   ) Anualmente

Há quanto tempo usa o iogurte ou leite fermentado? ....

Qual a marca do iogurte ou leite fermentado utilizado? ....

....

Utiliza suplementos à base de fibras atualmente? (   ) Não   (   ) Sim

Se sim, trata-se de:

(   ) Fibras solúveis   (   ) Mix de fibras/granola   (   ) Não sabe informar

Marca: ....

Qual a quantidade (g) consumida e há quanto tempo faz uso?

....

Com que frequência faz uso deste suplemento?

( ) 1x na semana ( ) 2 ou 3 x na semana ( ) Todos os dias ( ) Quinzenalmente ( ) Mensalmente ( ) A cada 3 meses ( ) Anualmente

Usa suplemento probiótico atualmente? ( ) Não ( ) Sim

Se sim, qual o suplemento utiliza? ....

Qual a frequência de uso?

( ) 1x na semana ( ) 2 ou 3 x na semana ( ) Todos os dias ( ) Quinzenalmente ( ) Mensalmente ( ) A cada 3 meses ( ) Anualmente

Há quanto tempo está usando o suplemento? ....

Pratica atividades físicas? ( ) Não ( ) Sim

Se sim, qual(is)? ....

Com que frequência?

( ) 1x na semana ( ) 2x na semana ( ) 3x na semana ( ) 4x na semana
( ) Diariamente

( ) Outro ....

Quanto tempo dedica a essa(s) atividade(s)? ....

Ingere alimentos nos intervalos das refeições? ( ) Não ( ) Sim

Se sim, qual(is)? ....

Hábitos intestinais (responder com o auxílio da imagem)

Frequência: ....

Consistência: ....

Escala de Bristol tipo: ....

Possui sangue nas fezes? (   ) Não   (   ) Sim

Possui dor ou desconforto abdominal? (   ) Não   (   ) Sim

Outras informações relevantes

Moradia em zona: (   ) Rural   (   ) Urbana

Fonte de água: (   ) Tratada   (   ) Poço   (   ) Outra ....

Possui animal de estimação? Qual? ....

Frequenta a creche? (   ) Sim   (   ) Não

Quanto tempo permanece lá? ....

Marca do Amido: ....

Observações:

....

....

....

....

....

Data da coleta:
/ /
Data de preenchimento do formulário:
/ /

Instruções para o preenchimento do registro alimentar de 3 dias

- Anotar o que foi ingerido em 3 dias: 2 dias da semana e 1 dia do final de semana. Preferencialmente não preencher nos feriados ou férias.
- As informações devem ser claras, constando também os métodos e ingredientes utilizados para a preparação dos alimentos, por exemplo: frito, assado ou cozido.
- A quantidade ingerida dos alimentos deve ser registrada, por exemplo: colher de sopa, sobremesa ou chá, xícara ou copo. Se possível, medir em mililitros (ml) os líquidos.
- Lembrar de escrever todos os condimentos, por exemplo: açúcar, maionese ou margarina, tempero para saladas, bem como os alimentos ingeridos fora dos horários das refeições.

Anotar sempre a quantidade ingerida, não a quantidade servida.

OBS: Circular o dia em que fez a coleta

ID:

Nº Protocolo:

DIA 01

Refeições	Local	Alimentos ingeridos e quantidade (colher, copo, xícara, mamadeira)
Café da manhã Horário:		
Lanche Horário:		
Almoço Horário:		
Lanche Horário:		
Jantar Horário:		

Intervalo das refeições	

Quantidade de amido (g)	Horário/Local	Misturado com

DIA 02

Refeições	Local	Alimentos ingeridos e quantidade (colher, copo, xícara, mamadeira)
Café da manhã Horário:		
Lanche Horário:		
Almoço Horário:		
Lanche Horário:		
Jantar Horário:		
Ceia Horário:		

Intervalo das refeições	

Quantidade de amido (g)	Horário/Local	Misturado com

DIA 03

Refeições	Local	Alimentos ingeridos e quantidade (colher, copo, xícara, mamadeira)
Café da manhã Horário:		
Lanche Horário:		
Almoço Horário:		
Lanche Horário:		
Jantar Horário:		
Ceia Horário:		

Intervalo das refeições	

Quantidade de amido	Horário/Local	Misturado com