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"Julio de Mesquita Filho"

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CARACTERIZAÇÃO DA MICROBIOTA INTESTINAL EM PACIENTES SUBMETIDOS AO TRANSPLANTE ALOGÊNICO DE MEDULA ÓSSEA E CORRELAÇÃO COM MARCADORES INFLAMATÓRIOS E DESFECHOS CLÍNICOS

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Botucatu
2026

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ALEXANDRE SOARES FERREIRA JUNIOR

Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração Biologia de micro-organismos e imunidade.

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Impacto Potencial Desta Pesquisa

Título da Tese: Caracterização Da Microbiota Intestinal em Pacientes Submetidos ao Transplante Alogênico De Medula Óssea e Correlação Com Marcadores Inflamatórios e Desfechos Clínicos.

Impacto Científico: Os achados deste projeto poderão contribuir para a identificação de marcadores capazes de prever complicações associadas ao transplante alogênico de medula óssea (alo-TMO), bem como apontar a microbiota intestinal como um possível alvo para intervenções terapêuticas, com o objetivo de reduzir a morbidade e a mortalidade relacionadas ao transplante.

Impacto Econômico e Social: Por se tratar de um estudo que avalia predominantemente pacientes atendidos pelo Sistema Único de Saúde (SUS), esta coorte representa uma população em situação de vulnerabilidade socioeconômica, frequentemente sub-representada em pesquisas conduzidas em países desenvolvidos. Os resultados obtidos poderão subsidiar futuras investigações voltadas ao desenvolvimento de terapias baseadas na modulação da microbiota intestinal, que apresentam potencial para maior acessibilidade.

Potential Impact of This Research

Thesis Title: Characterization of the Intestinal Microbiota in Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation and Its Correlation with Inflammatory Markers and Clinical Outcomes.

Scientific Impact: The findings of this project may contribute to the identification of markers capable of predicting complications associated with allo-HSCT, as well as to the recognition of the intestinal microbiota as a potential target for therapeutic interventions aimed at reducing transplantation-related morbidity and mortality.

Economic and Social Impact: Because this study predominantly evaluates patients from the Brazilian Unified Health System (SUS), the cohort represents a socioeconomically vulnerable population that is often underrepresented in research conducted in developed countries. The results may inform future studies aimed at developing therapies based on modulation of the gut microbiota, which have the potential for greater accessibility.

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Botucatu

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: Caracterização da microbiota intestinal em pacientes submetidos ao transplante alogênico de medula óssea e correlação com marcadores inflamatórios e desfechos clínicos

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Caracterização da Microbiota Intestinal em Pacientes Submetidos ao Transplante Alogênico de Medula Óssea e Correlação com Marcadores Inflamatórios e Desfechos Clínicos

Resumo

Em pacientes submetidos ao transplante alogênico de medula óssea (alo-TMO), há uma necessidade crescente de identificar novos fatores prognósticos e potenciais alvos terapêuticos capazes de melhorar os desfechos clínicos. Nesse contexto, a microbiota intestinal tem emergido como um determinante relevante, uma vez que estudos internacionais demonstram que alterações em sua diversidade e composição estão associadas a piores desfechos clínicos ao longo do alo-TMO. No entanto, dados provenientes da população brasileira ainda são escassos, e a maioria dos estudos disponíveis são observacionais, com limitada investigação dos mecanismos subjacentes a essas associações. O objetivo deste estudo é caracterizar a microbiota intestinal de pacientes brasileiros submetidos ao alo-TMO e investigar sua associação com marcadores inflamatórios, marcadores de permeabilidade intestinal e desfechos clínicos. Trata-se de um estudo prospectivo e multicêntrico, envolvendo quatro hospitais brasileiros localizados no estado de São Paulo. Foram incluídos pacientes com idade igual ou superior a 12 anos submetidos ao alo-TMO**.** A microbiota intestinal foi avaliada por sequenciamento do gene 16S rRNA, a permeabilidade intestinal por quantificação sérica de zonulina por ELISA, e as citocinas inflamatórias por citometria de fluxo. Ao todo, 267 pacientes contribuíram com 586 amostras de fezes e 877 amostras de sangue. As análises demonstram níveis persistentemente elevados de zonulina ao longo do alo-TMO, sugerindo aumento sustentado da permeabilidade intestinal. Além disso, a caracterização da microbiota intestinal revelou um perfil distinto de eventos de dominância intestinal na coorte brasileira**.** Adicionalmente, análises de correlação evidenciaram associações relevantes entre microbiota intestinal, permeabilidade intestinal e citocinas. Identificamos, por exemplo, correlações entre alguns gêneros bacterianos produtores de AGCC (ácidos graxos de cadeia curta) e os níveis de citocinas e zonulina, incluindo correlações negativas entre *Bifidobacterium* e os níveis de IL-6 e IFN-γ, e entre *Clostridia* e os níveis séricos de zonulina. Em conjunto, esses achados reforçam a relevância de uma abordagem integrada para a compreensão da interação entre microbiota intestinal, permeabilidade intestinal e citocinas no contexto do alo-TMO, destacando seu potencial como ferramenta prognóstica e como alvo para futuras intervenções terapêuticas**.**

Palavras-chave: Transplante alogênico de células-tronco hematopoiéticas; Doença do enxerto contra o hospedeiro; microbiota intestinal; e desfechos clínicos.

Characterization of the Intestinal Microbiota in Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation and Its Correlation with Inflammatory Markers and Clinical Outcomes

Abstract

In patients undergoing allogeneic bone marrow transplantation (allo-HSCT), there is a growing need to identify novel prognostic factors and potential therapeutic targets capable of improving clinical outcomes. In this context, the intestinal microbiota has emerged as a relevant determinant, as international studies have demonstrated that alterations in its diversity and composition are associated with worse outcomes throughout the allo-HSCT course. However, data derived from the Brazilian population remain scarce, and most available studies are observational, with limited investigation of the mechanisms underlying these associations. The aim of this study is to characterize the intestinal microbiota of patients undergoing allo-HSCT and to investigate its association with inflammatory markers, markers of intestinal permeability, and clinical outcomes. This is a prospective, multicenter study involving four Brazilian hospitals located in the state of São Paulo. Patients aged 12 years or older undergoing allo-HSCT were included. Intestinal microbiota was assessed by 16S rRNA gene sequencing, intestinal permeability was evaluated by serum zonulin quantification using ELISA, and inflammatory cytokines were measured by flow cytometry. In total, 267 patients contributed 586 stool samples and 877 blood samples. Preliminary analyses demonstrate persistently elevated zonulin levels throughout the allo-HSCT course, suggesting sustained increases in intestinal permeability. In addition, characterization of the intestinal microbiota revealed a distinct pattern of intestinal domination events in the Brazilian cohort. Additionally, correlation analyses revealed relevant associations between the gut microbiota, intestinal permeability, and cytokine levels. For example, we identified correlations between some SCFA-producing bacterial genera (short-chain fatty acids) and cytokine and zonulin levels, including negative correlations between *Bifidobacterium* and IL-6 and IFN-γ levels, and between *Clostridia* and serum zonulin levels. Taken together, these findings reinforce the relevance of an integrated approach to understanding the interplay between intestinal microbiota, intestinal permeability, and cytokines in the allo-HSCT setting, highlighting their potential as prognostic tools and as targets for future therapeutic interventions.

Keywords: allogeneic hematopoietic stem cell transplantation; Graft-versus-host disease; intestinal microbiota; and clinical outcomes.

SUMÁRIO

Ficha Catalográfica..... 3
Impacto Potencial Desta Pesquisa 4
Folha de Aprovação 5
Agradecimentos 6
Resumo 8
Atividades Acadêmicas 11
1. Introdução 24
2. Justificativa 29
3. Objetivos 30
4. Casuística, Materiais E Métodos 30
4.1 Delineamento do Estudo, Aspectos Éticos e Instituições Envolvidas 30
4.2 Recrutamento dos Pacientes 31
4.3 Coleta de Dados Clínicos e Amostras Biológicas 31
4.4 Extração De DNA 32
4.5 Sequenciamento Genético 16S rRNA e Fluxo de Análise em Bioinformática 33
4.6 Dosagem de Citocinas e Zonulina 34
5. Resultados e Discussão 34
5.1. Dados Clínicos e Características das Amostras Biológicas 35
5.2. Manuscritos de Revisão 38
5.2.1. Manuscrito I – Microbiota Intestinal no Alo-TMO 38
5.2.2. Manuscrito II – Permeabilidade Intestinal no Alo-TMO 77
5.3. Manuscritos Originais 148
5.3.1. Manuscrito I – Eventos de Dominância Intestinal no Alo-TMO 148
5.3.2. Manuscrito II – Avaliação de Zonulina no Alo-TMO 176
5.3.3. Manuscrito III – Crosstalk Microbiota, Permeabilidade e Citocinas 197
5. Conclusão Final 229
Referências Bibliográficas 232

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Premiações

1. Prêmio Jovem Pesquisador - Ricardo Pasquini. "Dynamics of Intestinal Domination in Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation", Sociedade Brasileira de Transplante de Medula Óssea e Terapia Celular, 2025.

2. Menção Honrosa ao trabalho intitulado "Caracterização da microbiota oral de pacientes submetidos ao transplante alogênico de medula óssea e correlação com dados clínicos", III Simpósio da LABIDS (UNESP - Campus Botucatu), 2025.

3. Prêmio de melhor apresentação de pôster na área de Microbiologia Médica Humana - V Congresso Paranaense de Microbiologia, Universidade Estadual de Londrina (UEL), 2025.

4. 1° Lugar em Apresentação Oral - Prêmio Shiduca Itow Jankevicius - Melhor Trabalho Científico no V Congresso Paranaense de Microbiologia, Universidade Estadual de Londrina (UEL), 2025.

5. Prêmio de melhor trabalho científico no Hemostasis & Thrombosis Research Society Scientific Symposium, 2023.

6. Travel Scholarship Award devido a excelência de trabalho científico no American Society for Apheresis Annual Meeting, 2022.

Apresentações modalidade orais em eventos científicos

1. **Soares Ferreira Junior, A.**; Hirai, W.Y.; Amanda Niz Alvarez, D.; da Silva Souza, L.; Machado, L.D.; Silva, N.L.; Oliveira da Silva, L.A.; Ramos, C.R.N.; Ciconelli, R.M.; Feliciano, J.V.P.; Colturato, I.; Barros, G.M.N.; Scheinberg, P.; de Oliveira, G.L.V. Caracterização da microbiota intestinal em pacientes submetidos ao transplante alogênico de medula óssea e correlação com marcadores inflamatórios e desfechos clínicos. In: **Simpósio Anual do Programa de Pós-graduação em Biologia Geral e Aplicada**, 2024, Botucatu/SP.

2. Alvarez, D. A. N**.; Ferreira-Jr, A**. S.; Alvarez, M. V. N.; Souza, L. S.; Hirai, W. Y.; Barros, G. M. N.; Colturato, I.; Feliciano, J. V. P.; Scheinberg, P.; De Oliveira, G. L. V. Caracterização do bacterioma oral de pacientes submetidos ao transplante alogênico de medula óssea e

correlação com dados clínicos. In: **Simpósio Anual do Programa de Pós-graduação em Biologia Geral e Aplicada**, 2024, Botucatu/SP.

3. Alvarez, D. A. N.**; Ferreira-Jr, A**. S.; Alvarez, M. V. N.; Souza, L. S.; Hirai, W. Y.; Barros, G. M. N.; Colturato, I.; Feliciano, J. V. P.; Scheinberg, P.; De Oliveira, G. L. V. Caracterização do bacterioma oral de pacientes submetidos ao transplante alogênico de medula óssea e correlação com dados clínicos. In: **V Congresso Paranaense de Microbiologia (UEL)**, 2025, Londrina/PR.

4. Alvarez, D. A. N.; **Ferreira-Jr, A. S**.; Alvarez, M. V. N.; Souza, L. S.; Hirai, W. Y.; Barros, G. M. N.; Colturato, I.; Feliciano, J. V. P.; Scheinberg, P.; De Oliveira, G. L. V. Caracterização do bacterioma oral de pacientes submetidos ao transplante alogênico de medula óssea e correlação com dados clínicos. In: **Simpósio Anual do Programa de Pós-graduação em Biologia Geral e Aplicada,** 2025, Botucatu/SP.

5. **Soares Ferreira Junior, A.**; Hirai, W.Y.; Amanda Niz Alvarez, D.; da Silva Souza, L.; Machado, L.D.; Silva, N.L.; Oliveira da Silva, L.A.; Ramos, C.R.N.; Ciconelli, R.M.; Feliciano, J.V.P.; Colturato, I.; Barros, G.M.N.; Scheinberg, P.; de Oliveira, G.L.V. Caracterização da microbiota intestinal em pacientes submetidos ao transplante alogênico de medula óssea e correlação com marcadores inflamatórios e desfechos clínicos. In: **Simpósio Anual do Programa de Pós-graduação em Biologia Geral e Aplicada**, 2025, Botucatu/SP.

6. **Soares Ferreira Junior, A**.; Hirai, W.Y.; Amanda Niz Alvarez, D.; da Silva Souza, L.; Machado, L.D.; Silva, N.L.; Oliveira da Silva, L.A.; Ramos, C.R.N.; Ciconelli, R.M.; Feliciano, J.V.P.; Colturato, I.; Barros, G.M.N.; Scheinberg, P.; de Oliveira, G.L.V. Dynamics of Intestinal Domination in Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation. Oral presentation: 29º Congresso da Sociedade Brasileira de Transplante de Medula Óssea (SBTMO). 21/08/2025. Brasilia, Brazil.

7. Silva, N.L.; **Soares Ferreira Junior, A**.; Souza, L.S.; Colturato, I.; Feliciano, F.V.P.; Barros, G.M.N.; Ciconelli, R.M.; Scheinberg, P.; de Oliveira, G.L.V. Increased intestinal permeability in patients submitted to allogeneic bone marrow transplantation. Apresentação oral: 28º

Congresso da Sociedade Brasileira de Transplante de Medula Óssea (SBTMO); 02/08/2024. Fortaleza, Brasil.

8. Rodrigues da Silva BF, **Soares Ferreira Junior A**, da Silva Souza L, Luiz da Silva J, Machado LD, Alvarez DAN, Yoshio Hirai W, Piccolo Feliciano JV, Colturato I, Maurício Navarro Barros G, Scheinberg P, Mesquita Ciconelli R, de Oliveira GLV. The Microbiota-Immune System Axis in Allogeneic Hematopoietic Stem Cell Transplantation. Apresentação oral: 30º Congresso da Sociedade Brasileira de Transplante de Medula Óssea (SBTMO); 19/08/2026. Salvador, Brasil.

Apresentações modalidade pôster em eventos científicos

1. **Soares Ferreira Junior, A**.; Colturato, I.; Barros, G.M.N.; Feliciano, J.V.P.; Oliveira, M.C.; de Oliveira, G.L.V. Characterization of the Intestinal Microbiota in Patients Undergoing Allogeneic Transplantation and Presenting with Acute Graft-versus-Host Disease. VI Simpósio de Biociências e Microbiologia. 2022, São José do Rio Preto, Brasil.

2. **Soares Ferreira Junior, A**.; Barros, G.M.N.; Colturato, I.; Feliciano, J.V.P.; Oliveira, M.C.; de Oliveira, G.L.V. Microbiota in Allogeneic Transplantation and Acute Graft-versus-Host Disease. 12th Probiotics, Prebiotics, New Foods, Nutraceuticals, Botanicals & Phytomedicals for Nutrition & Human and Microbiota Health. 2023. Rome, Italy.

3. Salustiano, L.P.Q.; **Soares Ferreira Junior, A**.; Penna, A.L.B.; de Oliveira, G.L.V. Assessment of the Intestinal Microbiota in Different Types of Diet and Cardiometabolic Risk. VII Simpósio de Biociências e Microbiologia do Rio Preto. 2023, São José do Rio Preto, Brasil.

4. Linares Silva, N.; **Soares Ferreira Junior, A**.; da Silva Souza, L.; Colturato, I.; Piccolo Feliciano, J.V.; Navarro Barros, G.; Scheinberg, P.; Lelis Vilela de Oliveira, G. Increased Intestinal Permeability in Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation. XXXVI Congresso de Iniciação Científica da UNESP. 2024, Botucatu, Brasil.

5. Silva, N.L.; Souza, L.S.; **Soares Ferreira Junior, A**.; Colturato, I.; Barros, G.M.N.; Feliciano, F.V.P.; Oliveira, M.C.; de Oliveira, G.L.V. Increased Intestinal Permeability in Patients

Submitted to Allogeneic Bone Marrow Transplantation. 21st International Congress of Mucosal Immunology (ICMI). 2024, Copenhagen, Denmark

6. Alvarez, D. A. N.; **Ferreira-Jr, A. S**.; Alvarez, M. V. N.; Souza, L. S.; Hirai, W. Y.; Barros, G. M. N.; Colturato, I.; Feliciano, J. V. P.; Scheinberg, P.; De Oliveira, G. L. V. Caracterização da microbiota oral de pacientes submetidos ao transplante alogênico de medula óssea e correlação com dados clínicos. In: III Simpósio da LABIDS 2025, Botucatu/SP.

7. Alvarez, D. A. N.; **Ferreira-Jr, A. S**.; Alvarez, M. V. N.; Souza, L. S.; Hirai, W. Y.; Barros, G. M. N.; Colturato, I.; Feliciano, J. V. P.; Scheinberg, P.; De Oliveira, G. L. V. Caracterização do bacterioma oral de pacientes submetidos ao transplante alogênico de medula óssea e correlação com dados clínicos. In: V Congresso Paranaense de Microbiologia (UEL) 2025, Londrina/PR.

8. Alvarez, D. A. N.; **Ferreira-Jr, A. S**.; Alvarez, M. V. N.; Souza, L. S.; Hirai, W. Y.; Barros, G. M. N.; Colturato, I.; Feliciano, J. V. P.; Scheinberg, P.; De Oliveira, G. L. V. Caracterização do bacterioma oral de pacientes submetidos ao transplante alogênico de medula óssea e correlação com dados clínicos. In: XIV Congresso de Biociências (CONGREBIO) 2025, Botucatu/SP.

9. **Ferreira-Junior, A. S**.; Alvarez, D. A. N.; Barros, G. M. N.; Feliciano, J. V. P.; Colturato, I.; Scheinberg, P.; Chao, N.; De Oliveira, Gislane L. V. Oral and gut bacteriome fingerprint in patients submitted to allogeneic hematopoietic stem cell transplantation. In: 19th International Congress of Immunology, 2025, Viena, Austria.

10. **Soares Ferreira Junior A**; Amanda Niz Alvarez D; da Silva Souza L; Machado LD; Silva NL; Feliciano JVP; Colturato I; Barros GMN; Scheinberg P; de Oliveira GLV. Short-Chain Fatty Acid-Producing Gut Microbiota: Dynamics and Impact on Intestinal Permeability. Sao Paulo, Brazil; and Clinical Outcomes During Allogeneic Hematopoietic Stem Cell Transplantation. In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular (HEMO 2025). 2025, São Paulo, Brasil.

11. Silva NL; **Soares Ferreira Junior A**; Da Silva Souza L; Amanda Niz Alvarez D; Silva FB; Colturato I; Feliciano JVP; Barros GMN; Scheinberg P; de Oliveira GLV. Prognostic Significance of Intestinal Permeability in Brazilian Patients Undergoing Allogeneic Stem Cell. In: Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular (HEMO 2025). 2025, São Paulo, Brasil.

12. Silva, N.L.; **Soares Ferreira Junior, A**.; da Silva Souza, L.; Amanda Niz Alvarez, D.; Silva, F.B.; Colturato, I.; Feliciano, J.V.P.; Barros, G.M.N.; Scheinberg, P.; de Oliveira, G.L.V. Dynamics of Intestinal Permeability in Patients Undergoing Allogeneic Stem Cell Transplantation. Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular (HEMO 2025); 2025, São Paulo, Brasil.

13. **Soares Ferreira Junior, A.**; Hirai, W.Y.; Amanda Niz Alvarez, D.; da Silva Souza, L.; Machado, L.D.; Silva, N.L.; Oliveira da Silva, L.A.; Ramos, C.R.N.; Ciconelli, R.M.; Feliciano, J.V.P.; Colturato, I.; Barros, G.M.N.; Scheinberg, P.; de Oliveira, G.L.V. Predictors of Intestinal Domination in Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation. 29º Congresso da Sociedade Brasileira de Transplante de Medula Óssea. 2025. Brasília, Brasil.

14. **Soares Ferreira Junior, A.**; Hirai, W.Y.; Amanda Niz Alvarez, D.; da Silva Souza, L.; Machado, L.D.; Silva, N.L.; Oliveira da Silva, L.A.; Ramos, C.R.N.; Ciconelli, R.M.; Feliciano, J.V.P.; Colturato, I.; Barros, G.M.N.; Scheinberg, P.; de Oliveira, G.L.V. The Prognostic Significance of Intestinal Domination in Allogeneic Hematopoietic Stem Cell Transplantation Varies According to the Dominant Genera and the Population Being Evaluated. 29º Congresso da Sociedade Brasileira de Transplante de Medula Óssea. 2025. Brasília, Brasil.

15. Rodrigues da Silva BF, **Soares Ferreira Junior A**, da Silva Souza L, Luiz da Silva J, Machado LD, Alvarez DAN, Yoshio Hirai W, Piccolo Feliciano JV, Colturato I, Maurício Navarro Barros G, Scheinberg P, Mesquita Ciconelli R, de Oliveira GLV. Early Cytokine Signatures Predict Clinical Outcomes After Allogeneic Hematopoietic Stem Cell Transplantation. Apresentação oral: 30º Congresso da Sociedade Brasileira de Transplante de Medula Óssea (SBTMO); 19/08/2026. Salvador, Brasil.

16. Rodrigues da Silva BF, **Soares Ferreira Junior A**, da Silva Souza L, Luiz da Silva J, Machado LD, Alvarez DAN, Yoshio Hirai W, Piccolo Feliciano JV, Colturato I, Maurício Navarro Barros G, Scheinberg P, Mesquita Ciconelli R, de Oliveira GLV. Cytokines Dynamics in Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation. Apresentação oral: 30º Congresso da Sociedade Brasileira de Transplante de Medula Óssea (SBTMO); 19/08/2026. Salvador, Brasil.

Resumos publicados em anais de eventos científicos

1. Silva, N.L.; **Soares Ferreira Junior, A.**; Souza, L.S.; Colturato, I.; Feliciano, F.V.P.; Barros, G.M.N.; Ciconelli, R.M.; Scheinberg, P.; de Oliveira, G.L.V. Increased Intestinal Permeability in Patients Submitted to Allogeneic Bone Marrow Transplantation. Journal of Bone Marrow Transplantation and Cellular Therapy, July 2024; 5(1): 61.

2. **Soares Ferreira Junior, A.**; Hirai, W.Y.; Amanda Niz Alvarez, D.; da Silva Souza, L.; Machado, L.D.; Silva, N.L.; Oliveira da Silva, L.A.; Ramos, C.R.N.; Ciconelli, R.M.; Feliciano, J.V.P.; Colturato, I.; Barros, G.M.N.; Scheinberg, P.; de Oliveira, G.L.V. Dynamics of Intestinal Domination in Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation. Journal of Bone Marrow Transplantation and Cellular Therapy, August 2025; 6(Supplement 2): 35–36.

3. **Soares Ferreira Junior, A.**; Hirai, W.Y.; Amanda Niz Alvarez, D.; da Silva Souza, L.; Machado, L.D.; Silva, N.L.; Oliveira da Silva, L.A.; Ramos, C.R.N.; Ciconelli, R.M.; Feliciano, J.V.P.; Colturato, I.; Barros, G.M.N.; Scheinberg, P.; de Oliveira, G.L.V. Predictors of Intestinal Domination in Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation. Journal of Bone Marrow Transplantation and Cellular Therapy, August 2025; 6(Supplement 2): 171.

4. **Soares Ferreira Junior, A.**; Hirai, W.Y.; Amanda Niz Alvarez, D.; da Silva Souza, L.; Machado, L.D.; Silva, N.L.; Oliveira da Silva, L.A.; Ramos, C.R.N.; Ciconelli, R.M.; Feliciano, J.V.P.; Colturato, I.; Barros, G.M.N.; Scheinberg, P.; de Oliveira, G.L.V. The Prognostic Significance of Intestinal Domination in Allogeneic Hematopoietic Stem Cell Transplantation Varies According to the Dominant Genera and the Population Being

Evaluated. Journal of Bone Marrow Transplantation and Cellular Therapy, August 2025; 6(Supplement 2): 180–183.

5. Silva, N.; **Soares Ferreira Júnior, A**.; Souza, L.S.; Alvarez, D.A.N.; Silva, B.F.; Colturato, I.; Feliciano, J.V.P.; Barros, G.N.; Scheinberg, P.; de Oliveira, G.L.V. Dynamics Of Intestinal Permeability In Patients Undergoing Allogeneic Stem Cell Transplantation. Hematology, Transfusion and Cell Therapy. 2025 Oct;47:105502.

6. Silva, N.; **Soares Ferreira Júnior, A**.; Souza, L.S.; Alvarez, D.A.N.; Silva, B.F.; Colturato, I.; Feliciano, J.V.P.; Barros, G.N.; Scheinberg, P.; de Oliveira, G.L.V. Prognostic Significance of Intestinal Permeability in Brazilian Patients Undergoing Allogeneic Stem Cell Transplantation. Hematology, Transfusion and Cell Therapy. 2025 Oct;47:105545.

7. **Soares Ferreira Junior A**; Amanda Niz Alvarez D; da Silva Souza L; Machado LD; Silva NL; Feliciano JVP; Colturato I; Barros GMN; Scheinberg P; de Oliveira GLV. Short-Chain Fatty Acid-Producing Gut Microbiota: Dynamics and Impact on Intestinal Permeability. Hematology, Transfusion and Cell Therapy. 2025 Oct;47:105560.

Revisor Convidado de Periódicos

1. 2024 – atual: Journal of Thrombosis and Haemostasis.

2. 2026 – atual: Journal of Clinical Apheresis.

3. 2026 – atual: Research and Practice in Thrombosis and Haemostasis.

Disciplinas cursadas

1. Tópicos Especiais em Biologia Geral e Aplicada (2025).

2. Morfologia, Fisiologia e Genética de Microrganismos Procariotos (2023).

3. Morfologia, Fisiologia e Genética de Microrganismos Eucarióticos (2023).

4. Interação Microbiota e Sistema Imune de Mucosas (2023).

5. Resposta Imunológica a Infecções Virais (2021).

Experiência docente

Participação no Programa de Atividades e Aperfeiçoamento em Docência no Ensino Superior (PAADES), na disciplina Imunologia Aplicada de responsabilidade do Departamento de Genética, Microbiologia e Imunologia, Setor de Microbiologia e Imunologia do Instituto de Biociências de Botucatu - IB UNESP, com carga horária de 75h em atividades de apoio à docência.

1. Introdução

Em pacientes submetidos ao transplante alogênico de medula óssea (alo-TMO), há a necessidade de identificar fatores prognósticos que possam ser utilizados para a estratificação dos pacientes, a predição do risco de complicações e o direcionamento de novas abordagens terapêuticas (De Molla et al., 2021; Peng et al., 2022; Soares Ferreira Júnior et al., 2025). Essas novas estratégias terapêuticas são necessárias porque, apesar dos avanços significativos no cuidado e no suporte a esses pacientes nas últimas décadas, o alo-TMO ainda permanece associado a elevada morbidade e a complicações potencialmente fatais (De Molla et al., 2021; Peng et al., 2022; Soares Ferreira Júnior et al., 2025). Dentre essas complicações, destacam-se as infecções, a recaída da doença de base e a doença do enxerto contra o hospedeiro aguda (DECH). A DECH, por exemplo, constitui a principal causa de mortalidade não relacionada à recaída e pode acometer até 70% dos pacientes, a depender de fatores como o regime de condicionamento empregado e o grau de compatibilidade HLA entre doador e receptor (Ferrara et al., 2009; Hill et al., 2021; Ilett et al., 2020; Jagasia et al., 2012). As infecções, por sua vez, são particularmente frequentes durante os períodos de neutropenia e estão associadas a elevadas taxas de mortalidade (Nesher; Rolston, 2019; Soares Ferreira Júnior et al., 2025). Nesse contexto, a microbiota intestinal tem sido reconhecida como uma variável potencialmente capaz tanto de predizer o risco de complicações quanto de constituir um alvo terapêutico (DeFilipp et al., 2019; Haak et al., 2018; Peled et al., 2020; Shouval et al., 2019).

O potencial prognóstico e terapêutico da microbiota intestinal no contexto do alo-TMO torna-se ainda mais relevante quando se considera a importância crescente dessa modalidade terapêutica no cenário mundial e nacional. O alo-TMO constitui uma terapia potencialmente curativa, amplamente empregada no tratamento de diversas doenças malignas e não malignas, incluindo leucemias agudas, síndromes de falência medular, doenças autoimunes e doenças genéticas (Greco et al., 2026). Dados recentes demonstram um aumento expressivo no número de alo-TMO realizados em diferentes regiões do mundo. Em publicação recente da *European Society for Blood and Marrow Transplantation* (EBMT), baseada em dados provenientes de 53 países, foram registrados 21.023 procedimentos de alo-TMO em países europeus no ano de 2024, representando um aumento de 2,6% em relação ao período anterior (Greco et al., 2026). De forma semelhante, dados do *Center for International Blood and Marrow Transplant Research* (CIBMTR) evidenciaram um crescimento substancial no número de alo-TMO realizados nos Estados Unidos, alcançando aproximadamente 10.000 procedimentos em 2023 (Spellman et al., 2025). Na América Latina, resultados de uma pesquisa conduzida pelo *Latin*

American Blood and Marrow Transplantation Group (LABMT), envolvendo 14 países, identificaram a realização de 2.196 transplantes alogênicos em 2018, correspondendo a um aumento de aproximadamente 80% no número de procedimentos realizados entre 2012 e 2018 (Correa et al., 2022). Em conjunto, esses dados evidenciam uma tendência consistente de crescimento no número de alo-TMO em escala global. Esse crescimento reforça a necessidade clínica da identificação de biomarcadores prognósticos e de novas estratégias terapêuticas capazes de otimizar os desfechos clínicos desses pacientes, entre as quais se destaca a modulação da microbiota intestinal.

A microbiota intestinal é composta por microrganismos essenciais para a manutenção da saúde humana, incluindo bactérias, fungos e vírus, que colonizam todo o trato gastrointestinal e coexistem em simbiose com o hospedeiro (veja **Figura 1A**). Ela desempenha papel central na fisiologia humana, contribuindo para: (1) a homeostase do hospedeiro, (2) o metabolismo de nutrientes, (3) a síntese de vitaminas, (4) a modulação do sistema imunológico e (5) a produção de metabólitos (Fujiwara, 2021; Lynch; Pedersen, 2016; Soares Ferreira Júnior et al., 2025). Em virtude de suas múltiplas funções e de suas complexas interações com o organismo humano, a microbiota intestinal tem sido reconhecida como uma variável relevante na patogênese de diversas doenças. Por exemplo, a microbiota intestinal vem sendo associada à doença inflamatória intestinal, à síndrome do intestino irritável, a doenças autoimunes e a distúrbios metabólicos (Lynch; Pedersen, 2016; Parlato et al., 2018; Qiu et al., 2022). Além disso, a microbiota intestinal vem sendo investigada como ferramenta auxiliar no diagnóstico de neoplasias e como fator preditor de resposta a quimioterápicos e imunoterapias (Lei et al., 2025; Nobels et al., 2025). Diante de seu potencial como variável prognóstica e como alvo terapêutico, torna-se necessária uma melhor compreensão da microbiota intestinal no contexto do alo-TMO.

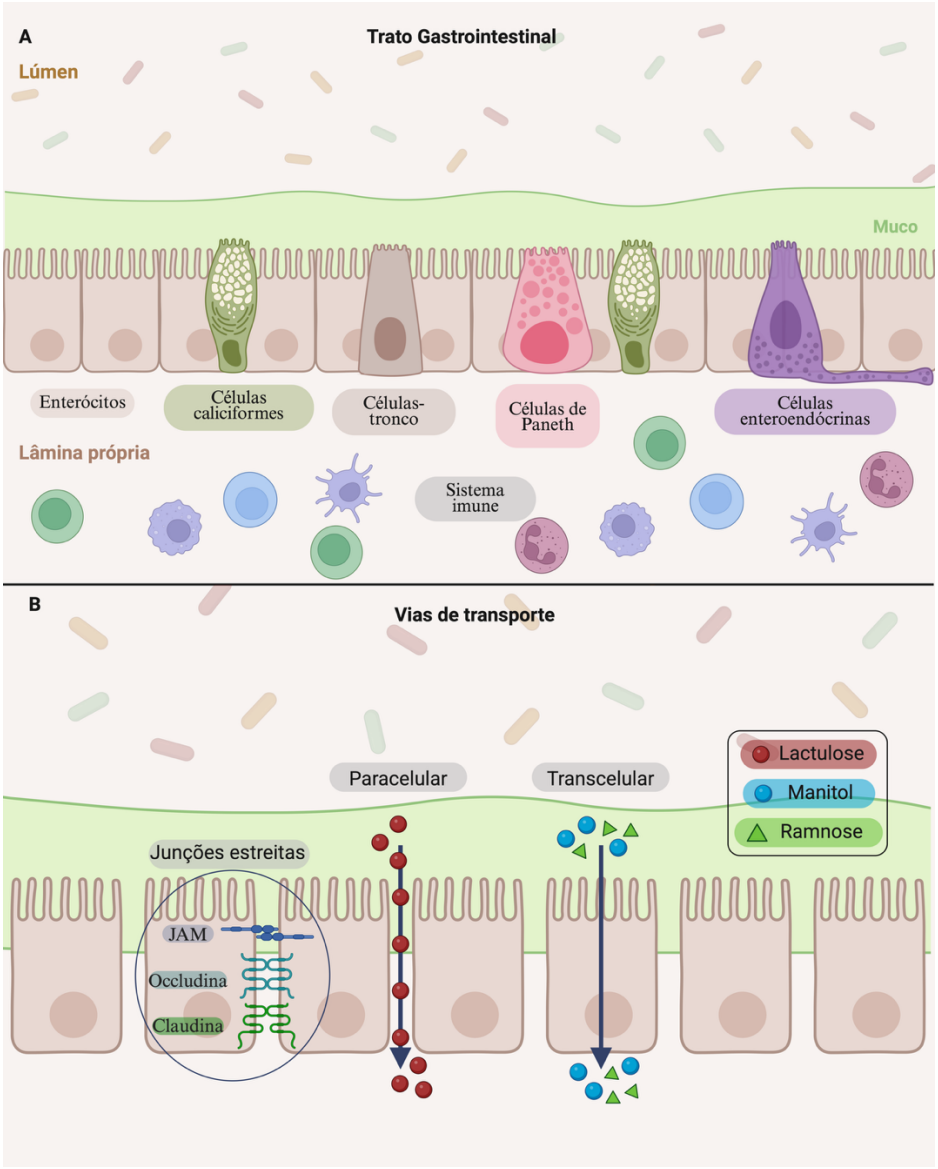


Figura 1. A) Barreira gastrointestinal saudável e suas três camadas (camada de muco, epitélio intestinal e lâmina própria). B) Vias de transporte paracelular e transcelular através do epitélio intestinal. Criado no BioRender. Soares Ferreira Junior, A. (2026). Criada em https://BioRender.com.

No contexto do alo-TMO, estudos internacionais já têm demonstrado achados relevantes acerca do papel da microbiota intestinal (Haak et al., 2018; Messina et al., 2024; Peled et al., 2020; Taur et al., 2012). Padrões específicos de alterações na composição da microbiota intestinal têm sido associados a desfechos clínicos relacionados ao alo-TMO. A diminuição da diversidade intestinal, por exemplo, tem sido associada a maior incidência de DECH e a pior sobrevida global (Liu et al., 2017; Messina et al., 2024; Peled et al., 2020). Alterações na composição da microbiota intestinal, como a diminuição de gêneros produtores de ácidos

graxos de cadeia curta (metabólito intestinal importante para manutenção da integridade da barreira intestinal), vem sendo associadas a maior incidência de infecções de vias aéreas e diminuição da sobrevida global (Biagi et al., 2015; Du et al., 2024; Haak et al., 2018; Meedt et al., 2022; Romick-Rosendale et al., 2018). Apesar desses achados provenientes de coortes internacionais, ainda são escassos os estudos que avaliam o papel da microbiota intestinal na população brasileira. Dessa forma, torna-se necessária uma melhor compreensão da influência da microbiota intestinal nesse contexto em nossa população.

Além disso, a maioria dos estudos sobre microbiota intestinal é de natureza observacional, o que limita a realização de análises mais aprofundadas capazes de elucidar os mecanismos pelos quais a microbiota exerce influência sobre o hospedeiro (Soares Ferreira Júnior et al., 2025). Na ausência dessa compreensão mecanista, frequentemente permanece a dúvida quanto à natureza causal dessas associações, isto é, se as alterações da microbiota intestinal constituem a causa ou a consequência de determinados desfechos clínicos (Buttó; Haller, 2016; Soares Ferreira Júnior et al., 2025). Dessa forma, tornam-se necessários estudos que não apenas caracterizem a composição da microbiota intestinal, mas que também investiguem os possíveis mecanismos pelos quais ela pode modular a fisiologia do ser humano. Entre os principais mecanismos frequentemente abordados destacam-se o aumento da permeabilidade intestinal e a interação com o sistema imunológico (Fiorenza; Turtle, 2021; Schluter et al., 2020; Skaarud et al., 2021; Tyszka et al., 2022; Wang et al., 2023).

A permeabilidade intestinal refere-se a um conceito complexo (veja **Figura 1B**), determinado pela integridade e pela interação entre os diversos componentes que constituem a barreira do trato gastrointestinal (Dmytriv; Storey; Lushchak, 2024). A barreira gastrointestinal é uma estrutura física com propriedades de permeabilidade seletiva (transporte controlado), impedindo entrada de substâncias nocivas e patógenos. Ela é constituída principalmente por três camadas: (1) a camada de muco, incluindo as subcamadas interna e externa; (2) o epitélio intestinal; e (3) a lâmina própria, que contém células do sistema imunológico (Dmytriv; Storey; Lushchak, 2024). A camada de muco é formada predominantemente por mucinas e por microrganismos comensais, incluindo bactérias, fungos, vírus e parasitas. Já o epitélio intestinal é formado por uma monocamada de células especializadas, incluindo enterócitos, células caliciformes, células de Paneth, células enteroendócrinas e células M (microfold) (Dmytriv; Storey; Lushchak, 2024). Por fim, a terceira camada, a lâmina própria, é constituída por componentes do sistema imunológico entérico, incluindo macrófagos e células dendríticas (Dmytriv; Storey; Lushchak, 2024).

Durante o alo-TMO, diversos fatores podem comprometer a integridade da barreira do trato gastrointestinal e levar ao aumento da permeabilidade intestinal, incluindo: (1) o regime de condicionamento, (2) alterações na dieta e (3) modificações na composição da microbiota intestinal (Blijlevens et al., 2005; Johansson; Ekman, 2007; Rashidi et al., 2018; Skaarud et al., 2021; Van der Velden et al., 2010; Wang et al., 2023). A disfunção dessa barreira favorece a translocação de metabólitos, patógenos e componentes microbianos (padrões moleculares associados a dano e a patógenos [DAMPS/PAMPS]) para a lâmina própria e para a circulação sistêmica, com consequente ativação de células do sistema imunológico. Esse processo tem sido proposto como um dos principais mecanismos pelos quais a microbiota intestinal pode influenciar os desfechos clínicos no contexto do alo-TMO. De fato, estudos demonstram associações importantes entre o aumento da permeabilidade intestinal e piores desfechos ao longo do transplante (Johansson; Ekman, 2007; Tyszka et al., 2022; Ye et al., 2012), reforçando a necessidade de investigações que avaliem de forma integrada a microbiota intestinal e a integridade da barreira intestinal.

Outro mecanismo pelo qual a microbiota intestinal pode desempenhar papel relevante na modulação dos desfechos é por meio de sua interação com o sistema imunológico (Fiorenza; Turtle, 2021; Wolfe; Markey, 2022). Essa associação torna-se ainda mais pertinente no contexto do alo-TMO, no qual o processo de reconstituição imune após o regime de condicionamento é fundamental para o sucesso do transplante (Fiorenza; Turtle, 2021; Wolfe; Markey, 2022). Esse processo de reconstituição imune promove o desenvolvimento de um novo sistema imunológico inato e adaptativo, essencial para a prevenção da recaída da doença de base e para o controle de infecções (efeito enxerto versus tumor) (Fiorenza; Turtle, 2021; Wolfe; Markey, 2022). Contudo, essa mesma reconstituição imune pode também contribuir para o desenvolvimento da DECH e outros desfechos clínicos (Fiorenza; Turtle, 2021; Wolfe; Markey, 2022). Por exemplo, enquanto a recuperação mais rápida de neutrófilos e de células NK foi associada, em estudos prévios, a maior risco de DECH, a reconstituição precoce de células MAIT e de células B tem sido relacionada a um menor risco dessa complicação (Ando et al., 2020; Kawaguchi et al., 2018; Minculescu et al., 2020; Nagayama et al., 2020; Van der Maas et al., 2019). Essa reconstituição imune constitui, portanto, um importante fator prognóstico ao longo do alo-TMO. Além disso, no alo-TMO, estudos têm demonstrado que padrões específicos da composição da microbiota intestinal vêm sendo associados a marcadores da resposta imune. Por exemplo, o aumento de gêneros como *Faecalibacterium, Ruminococcus* e *Akkermansia* foi associado à recuperação de neutrófilos, enquanto maior diversidade alfa tem sido relacionada a

contagens mais elevadas de linfócitos T $CD4^+$ e $CD8^+$ (Miltiadous et al., 2022; Schluter et al., 2020). Esses e outros achados evidenciam o potencial impacto clínico de uma melhor caracterização da interação entre a microbiota intestinal e o sistema imunológico no contexto do alo-TMO.

No presente estudo, o objetivo foi realizar uma avaliação abrangente e crítica do papel da microbiota intestinal em pacientes brasileiros submetidos ao alo-TMO. Adicionalmente, propomos investigar marcadores inflamatórios e marcadores de permeabilidade intestinal, contemplando dois dos principais mecanismos por meio dos quais a microbiota intestinal pode influenciar os desfechos clínicos. Nos capítulos subsequentes desta tese, serão apresentados a justificativa, os objetivos, a casuística e os métodos empregados no desenvolvimento do projeto. A apresentação dos resultados será realizada por meio dos artigos científicos decorrentes deste estudo, os quais já foram publicados ou submetidos para revisão de pares até a presente data.

2. Justificativa

Apesar dos avanços significativos da medicina nas últimas décadas, o alo-TMO ainda está associado a elevada morbimortalidade, principalmente em decorrência de complicações como infecções, DECH e mortalidade relacionada ao transplante. Nesse contexto, torna-se essencial o desenvolvimento de estratégias que possibilitem melhor predição de riscos e a otimização do manejo clínico dos pacientes ao longo do procedimento. Embora o papel da microbiota intestinal tenha sido amplamente investigado em coortes internacionais, ainda são escassos os dados sobre sua atuação e influência na população brasileira. Além disso, são limitados os estudos mecanísticos que avaliam os processos pelos quais a microbiota intestinal pode influenciar o desenvolvimento dessas complicações, incluindo alterações na permeabilidade intestinal e na modulação da resposta imune. Essas lacunas são particularmente relevantes ao se considerar que a microbiota intestinal é fortemente influenciada por fatores sociogeográficos, ambientais e culturais. Dessa forma, o presente projeto justifica-se pelos seguintes pontos:

- Escassez de dados em populações brasileiras, especialmente em pacientes atendidos pelo Sistema Único de Saúde (SUS), ressaltando a necessidade de estudos conduzidos em contextos locais.
- Necessidade de identificar biomarcadores preditivos capazes de monitorar e prever desfechos clínicos adversos ao longo das diferentes fases do alo-TMO na população brasileira.

- Possibilidade de utilização da microbiota intestinal como alvo terapêutico, por meio de intervenções destinadas à sua modulação, com o objetivo de reduzir a morbidade e a mortalidade relacionadas ao alo-TMO
- Limitação do conhecimento mecanístico acerca da disbiose intestinal e de seus efeitos sobre a permeabilidade intestinal e a modulação da resposta imune no alo-TMO.

3. Objetivos

3.1 Objetivo Geral

Caracterizar a microbiota intestinal de pacientes submetidos ao alo-TMO e investigar sua associação com marcadores inflamatórios, marcadores de permeabilidade intestinal e desfechos clínicos.

3.2 Objetivos Específicos

1. Construir um banco de dados sociodemográficos e clínicos, bem como um banco de amostras biológicas (fezes e sangue), de pacientes brasileiros submetidos ao alo-TMO.
2. Identificar perfis ("fingerprints") da microbiota intestinal relevantes para a população brasileira, com potencial utilização como marcadores preditivos de desfechos clínicos.
3. Caracterizar a dinâmica da microbiota intestinal ao longo do alo-TMO e identificar fatores preditores associados às alterações observadas.
4. Caracterizar a dinâmica da permeabilidade intestinal ao longo do alo-TMO e avaliar sua associação com a microbiota intestinal.
5. Avaliar a associação entre a microbiota intestinal e marcadores inflamatórios sistêmicos.

4. Casuística, Materiais E Métodos

4.1 Delineamento do Estudo, Aspectos Éticos e Instituições Envolvidas

Este é um estudo multicêntrico, observacional, prospectivo e de coorte que foi aprovado pelo Comitê de Ética em Pesquisa da Universidade Estadual Paulista (Processo n° 5.138.190/2021). Todos os participantes incluídos no trabalho assinaram o termo de assentimento ou consentimento livre e esclarecido. Os responsáveis legais pelos menores de 18 anos também assinaram termo específico para os representantes legais. Foram incluídos

pacientes com idade igual ou superior a 12 anos submetidos ao alo-TMO em uma das instituições participantes.

As instituições colaboradoras no recrutamento de pacientes foram a Fundação Faculdade Regional de Medicina de São José do Rio Preto (FUNFARME), Fundação Pio XII – Hospital de Câncer de Barretos (HCB), Fundação Doutor Amaral Carvalho de Jaú (FACJ) e Hospital Beneficência Portuguesa de São Paulo (BP). O projeto foi aprovado nos comitês de ética locais de cada uma das instituições incluídas como coparticipantes na Plataforma Brasil.

4.2 Recrutamento dos Pacientes

Foram incluídos no estudo pacientes submetidos ao alo-TMO com idade igual ou superior a 12 anos, independentemente do grau de compatibilidade HLA, e que apresentassem acompanhamento médico regular, com pelo menos uma avaliação médica após o transplante. Foram excluídos pacientes com idade inferior a 12 anos, aqueles submetidos ao transplante autólogo de medula óssea e os que receberam transplante de células-tronco provenientes de sangue de cordão umbilical.

4.3 Coleta de Dados Clínicos e Amostras Biológicas

Todos os dados sociodemográficos e clínicos relacionados a este projeto foram registrados na plataforma REDCap (Research Electronic Data Capture), um sistema web seguro destinado à criação, ao gerenciamento e à disseminação de dados em pesquisas científicas.

Para a coleta das informações clínicas e sociodemográficas, foram utilizados quatro formulários padronizados. O Formulário 1 contemplou as características sociodemográficas dos participantes, incluindo sexo, idade, raça/cor e grau de escolaridade. O Formulário 2 reuniu dados referentes ao procedimento de alo-TMO, tais como indicação para o transplante, data da infusão, tipo de regime de condicionamento e grau de compatibilidade HLA. O Formulário 3 incluiu informações sobre as principais complicações clínicas associadas ao alo-TMO, como infecções, DECH aguda, recaída da doença de base e óbito. Por fim, o Formulário 4 foi destinado à coleta de dados detalhados sobre a DECH aguda, abrangendo os órgãos acometidos, os escores de gravidade (MAGIC score) e as informações referentes ao tratamento instituído. Os formulários podem ser encontrados nos Anexos desse documento.

Em relação à coleta de amostras biológicas, foram estabelecidos seis períodos fixos: pré-condicionamento (D−7), dia da infusão de células (D0), 30 dias (D+30), 60 dias (D+60), 90 dias

(D+90) e 180 dias (D+180) após o alo-TMO. Adicionalmente, amostras foram coletadas no momento da suspeita clínica de DECH aguda.

As amostras de sangue periférico foram obtidas em tubos com gel separador (8,5 mL) ou em dois tubos de 5 mL, sendo inicialmente armazenadas nos centros colaboradores a −20 °C até o transporte para a instituição sede (Instituto de Biociências de Botucatu – UNESP), onde foram posteriormente mantidas a −80 °C. As amostras de fezes foram coletadas em frascos estéreis de 50 a 80 mL e igualmente armazenadas a −20 °C nos centros de origem, sendo posteriormente transportadas e conservadas a −80 °C na instituição sede para análises posteriores.

4.4 Extração De DNA

A extração de DNA foi realizada utilizando o kit comercial Fast DNA Stool Mini Kit (QIAGEN, Alemanha), de acordo com o protocolo do fabricante. Inicialmente, na etapa de suspensão, foram adicionados 2 mL de InhibitEX Buffer aos tubos Falcon contendo 200mg de amostras fecais, seguidos de incubação por 5 minutos à temperatura ambiente (25 °C). Após esse período, as amostras foram homogeneizadas em vórtex por 1 minuto.

Em seguida, os tubos foram submetidos a banho-maria por 5 minutos a 95 °C, com o objetivo de promover a lise de bactérias Gram-positivas, e posteriormente homogeneizados novamente em vórtex por 15 segundos. As amostras foram então centrifugadas por 10 minutos a 25 °C, em velocidade máxima, para a formação do pellet.

Na etapa de digestão, 600 µL do sobrenadante foram transferidos para um tubo de microcentrífuga de 2 mL, aos quais foram adicionados 25 µL de Proteinase K, com homogeneização por pipetagem (*up-down*). Em seguida, foram acrescentados 600 µL de Buffer AL, com homogeneização em vórtex por 15 segundos. As amostras foram incubadas em banho-maria por 10 minutos a 70 °C, sendo posteriormente adicionados 600 µL de etanol absoluto (100%), seguidos de nova homogeneização em vórtex por 15 segundos.

Na etapa de transferência, alíquotas de 500 µL da solução foram aplicadas na coluna de sílica e submetidas à centrifugação por 1 minuto em velocidade máxima. O tubo coletor foi então substituído, descartando-se o eluato, e o procedimento foi repetido aproximadamente três vezes, até que todo o volume da amostra fosse processado.

Na etapa de lavagem, foram adicionados 500 µL de Buffer AW1 à coluna, seguidos de centrifugação por 1 minuto em velocidade máxima e troca do tubo coletor. Em seguida, foram adicionados 500 µL de Buffer AW2, com centrifugação por 3 minutos em velocidade máxima e nova substituição do tubo coletor. Para completa remoção de resíduos, as colunas foram

submetidas a uma centrifugação adicional por 3 minutos em velocidade máxima, sendo então transferidas para tubos de microcentrífuga de 2 mL estéreis.

Por fim, na etapa de eluição, foram adicionados 50 µL de Buffer ATE diretamente à membrana da coluna, seguidos de incubação por 1 minuto à temperatura ambiente (25 °C) e centrifugação por 1 minuto em velocidade máxima. Após a eluição, as colunas foram descartadas, e o DNA obtido foi armazenado para as análises subsequentes.

4.5 Sequenciamento Genético 16S rRNA e Fluxo de Análise em Bioinformática

O DNA foi quantificado utilizando o Qubit dsDNA HS Assay Kit (Thermofisher, Waltham, MA, EUA). As regiões V3–V4 do rRNA 16S foram então amplificadas em um protocolo de PCR em duas etapas, utilizando os seguintes primers previamente determinados e contendo adaptadores Illumina tipo Nextera — 341F: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG e 805R: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC. As reações de PCR utilizaram o 2X Platinum SuperFi II PCR Master Mix (Thermofisher) e envolveram uma etapa de desnaturação a 98 °C por 30 s, seguida de 30 ciclos de: 98 °C por 10 s, 55 °C por 10 s e 72 °C por 30 s. O sequenciamento foi realizado na plataforma Illumina NextSeq 2000 (San Diego, CA, EUA). Um total de 108.251.088 leituras foi gerado (média de 217.371 por amostra) e submetido à remoção de adaptadores usando o Cutadapt v2.6 (Martin, 2011). Em seguida, as leituras passaram por filtragem de qualidade, remoção de ruído, remoção de quimeras e inferência de variantes de sequência de amplicons (ASVs) utilizando o DADA2 v.1.26.0 (Callahan et al., 2016). Um total de 59.994.735 leituras (média de 120.471 por amostra) passou por todas as etapas de filtragem e foi utilizado nas análises subsequentes.

A classificação taxonômica foi realizada utilizando o classificador Naive Bayes do Classify-sklearn, conforme implementado no Qiime2 (plugin q2-feature-classifier), treinado com o banco de dados SILVA SSU v138.1 NR 99 (Bokulich et al., 2018; Bolyen et al., 2019). A abundância relativa das diferentes ASVs em cada amostra foi analisada no Qiime2 e representada graficamente com o plugin taxa (função barplot). Os índices de diversidade alfa e beta, bem como os testes estatísticos para diferenças significativas entre os grupos experimentais, foram calculados utilizando o plugin diversity do Qiime2. O método de normalização utilizado no estudo foi a rarefação para 30.000 leituras por amostra (plugin diversity do Qiime2, função alpha-rarefaction). Foram elaboradas tabelas para calcular a

abundância de leituras atribuídas a cada ASV, que posteriormente foram agregadas ao nível de gênero para as análises. Os dados atualmente disponíveis, incluindo informações clínicas e dados provenientes de experimentos moleculares, já foram depositados no repositório institucional da universidade: Repositório Institucional UNESP (https://hdl.handle.net/11449/323828). Entretanto, considerando que novas análises ainda estão em andamento, a disponibilização pública antecipada dos dados poderia comprometer a integridade científica das análises em curso. Dessa forma, os dados serão tornados públicos somente após a conclusão de todas os artigos em andamento. Os dados de sequenciamento genético já disponíveis foram depositados no NCBI Sequence Read Archive sob o BioProject PRJNA1357096, permanecendo temporariamente sob acesso restrito até a finalização das análises.

4.6 Dosagem de Citocinas e Zonulina

Aproximadamente 5 mL de sangue periférico foram coletados dos participantes em tubos contendo EDTA K2, e o plasma foi separado por centrifugação a 1372× g por 10 minutos a 4 °C. As amostras de plasma foram utilizadas para a quantificação de citocinas e de zonulina. Para a quantificação de citocinas, foi empregada a técnica de cytometric bead array (Human Th1/Th2/Th17 Kit, BD Biosciences, San Jose, CA, EUA). Os níveis de interleucinas (IL)-2, IL-4, IL-6, IL-10, IL-17A, interferon-gama (IFN-γ) e fator de necrose tumoral (TNF) foram determinados em citômetro de fluxo (FACSCanto™ II, BD Biosciences, Franklin Lakes, NJ, EUA), com análise realizada no software BDFCAP Array™ v3 e resultados expressos em pg/mL. A citocina fator de crescimento transformador beta (TGF-β) foi quantificada por meio do kit ELISA sanduíche TGF-β1 (Elabscience, Bethesda, MD, EUA), de acordo com as instruções do fabricante, com leitura de absorbância a 450 nm e resultados expressos em ng/mL. Para a quantificação de zonulina plasmática, foi utilizado o kit Human Zonulin ELISA (Elabscience, Bethesda, MD, EUA), conforme as recomendações do fabricante.

5. Resultados e Discussão

Os resultados deste trabalho de doutorado serão apresentados predominantemente na forma de artigos científicos, incluindo manuscritos já publicados e aqueles elaborados e submetidos à revisão por pares, os quais constituem os principais produtos deste projeto. Além disso, será incluída uma seção inicial com a caracterização geral da coorte, contemplando dados clínicos dos pacientes e informações sobre a coleta e o processamento das amostras biológicas.

Os manuscritos de revisão fornecerão a fundamentação teórica do estudo, enquanto os artigos originais apresentarão e discutirão os achados experimentais e clínicos obtidos ao longo do doutorado.

5.1. Dados Clínicos e Características das Amostras Biológicas

Ao longo desse projeto, 271 pacientes foram recrutados (veja Figura 2). Destes, quatro (1.5%) optaram por sair do estudo. A Tabela 1 apresenta as características sociodemográficas e clínicas da coorte deste estudo, composta por 267 pacientes submetidos ao alo-TMO nos quatro centros participantes. Como este projeto de doutorado faz parte de um estudo multicêntrico maior, cujo recrutamento de pacientes permanece em andamento, nem todos os participantes incluídos até o momento completaram o período de seguimento (*follow-up*). Dessa forma, embora a coorte conte atualmente com 267 pacientes, alguns ainda não possuem dados completos para todas as variáveis analisadas. A idade média da população no momento do alo-TMO foi de 42 ± 16 anos, com discreto predomínio do sexo masculino (58%). As principais indicações para o alo-TMO corresponderam às leucemias agudas (64%). O regime de condicionamento mais frequentemente empregado foi o de intensidade reduzida (45%), e a principal fonte de células-tronco hematopoéticas foi o sangue periférico (82%).

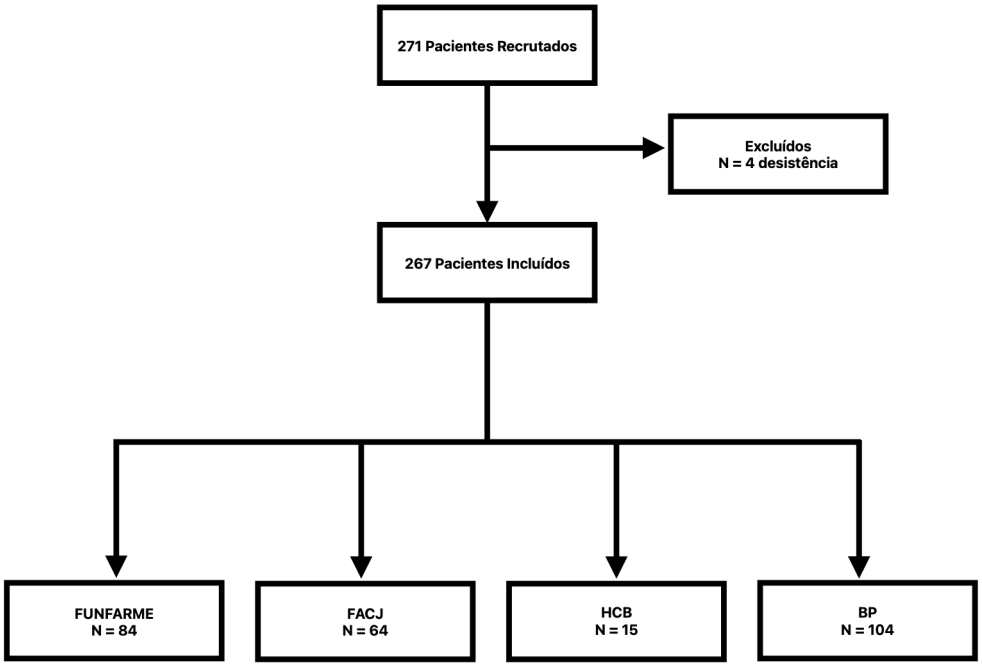


Figura 2. Diagrama dos pacientes inseridos nesse estudo. N = Número de pacientes; BP = Beneficência Portuguesa; FUNFARME = Fundação Faculdade Regional de Medicina de São José do Rio Preto; FACJ = Fundação Amaral Carvalho de Jau; HCB = Hospital de Câncer de Barretos.

Tabela 1. Características sociodemográficas dos pacientes incluídos*

	FUNFARME (N = 84)	FACJ (N = 64)	HCB (N = 15)	BP (N = 104)	Total (N = 267)
Idade (anos)					
Média (DP)	39 (15)	41 (15)	40 (14)	45 (18)	42 (16)
Mediana (Min-Max)	38 (15-67)	41 (13-68)	37 (19-61)	43 (12-73)	41 (12-73)
Gênero					
Masculino	50 (60%)	40 (63%)	8 (53%)	56 (54%)	154 (58%)
Feminino	34 (40%)	24 (38%)	7 (47%)	48 (46%)	113 (43%)
Peso (Kg)					
Média (DP)	78.5 (16.9)	76.1 (15.0)	71.5 (14.7)	74.1 (17.1)	75.9 (16.5)
Mediana (Min-Max)	77.8 (47.8-131.4)	74.0 (47-108.7)	75 (43-94.7)	73 (23-130.0)	74 (23-131.4)
Altura (cm)					
Média (DP)	169.6 (9.8)	168.0 (10.4)	167.3 (12.2)	167.9 (10.5)	168.5 (10.4)
Mediana (Min-Max)	170.5 (150-192)	167 (144-195)	168 (146-189)	168 (124-189)	168 (124-195)
IMC					
Média (DP)	27.3 (5.6)	26.9 (4.5)	25.0 (5.2)	26.1 (4.6)	26.6 (5.0)
Mediana (Min-Max)	26.9 (15.8-48.3)	26.2 (16.9-37.6)	23.5 (17.5-38.0)	25.4 (15.0-38.4)	25.9 (15.0-48.3)
Cor					
Caucasianos	60 (71%)	28 (44%)	9 (60%)	68 (69%)	165 (63%)
Afrodescentens	23 (27%)	36 (56%)	6 (40%)	28 (29%)	93 (36%)
Asiáticos	1 (1%)	0 (0%)	0 (0%)	2 (2%)	3 (1%)
Indicação Alo-TMO					
LMA	28 (42%)	22 (34%)	5 (33%)	41 (42%)	96 (40%)
LMC	5 (8%)	5 (8%)	0 (0%)	1 (1%)	11 (5%)
LLA	15 (23%)	16 (25%)	4 (27%)	23 (24%)	58 (24%)
LLC	1 (2%)	1 (1.6%)	0 (0%)	0 (0%)	2 (1%)
LH	3 (5%)	1 (1.6%)	1 (6.7%)	5 (5%)	10 (4%)
LNH	0 (0%)	0 (0%)	2 (13%)	3 (3%)	5 (2%)
Anemia Aplástica	8 (12%)	4 (6%)	0 (0%)	3 (3%)	15 (6%)
Anemia Falciforme	2 (3%)	0 (0%)	1 (7%)	2 (2%)	5 (2%)
Outros	4 (6%)	15 (23%)	2 (13%)	19 (20%)	40 (17%)
Condicionamento					
Mieloablativo	24 (35%)	30 (47%)	4 (29%)	21 (22.6%)	79 (33%)
Não Mieloablativo	7 (12%)	2 (3.1%)	0 (0%)	42 (45.2%)	51 (22%)
Intensidade Reduzida	35 (53%)	32 (50%)	10 (71%)	30 (32.2%)	107 (45%)
Fonte de Células					

Sangue Periférico	75 (91%)	40 (63%)	13 (93%)	82 (85.4%)	210 (82%)
Medula Óssea	6 (7%)	24 (38%)	1 (7.1%)	10 (10.4%)	41 (16%)
Outras	1 (1%)	0 (0%)	0 (0%)	4 (4.2%)	5 (2%)
Sexo do Doador					
Masculino	49 (59%)	46 (73%)	9 (64%)	59 (61%)	164 (64%)
Feminino	33 (41%)	17 (27%)	5 (36%)	36 (38%)	91 (36%)
Compatibilidade					
MRD	29 (44%)	20 (31%)	2 (14%)	27 (28.1%)	78 (33%)
MUD	2 (3%)	10 (16%)	3 (21%)	13 (13.5%)	28 (12%)
Haploidêntico	35 (53%)	30 (47%)	7 (50%)	43 (44.8%)	115 (48%)
MMUD	0 (0%)	4 (6.3%)	0 (0%)	5 (5.2%)	9 (4%)
Aparentado com mismatch HLA 5-10	0 (0%)	0 (0%)	2 (14%)	7 (7.3%)	9 (4%)
Outros	0 (0%)	0 (0%)	0 (0%)	1 (1.0%)	1 (0.4%)

Alo-TMO = Transplante alogênico de medula óssea; BP = Beneficência Portuguesa; Cm = centímetros; DP = Desvio padrão; FUNFARME = Fundação Faculdade Regional de Medicina de São José do Rio Preto; FACJ = Fundação Amaral Carvalho de Jau; Kg = quilogramas; HCB = Hospital de Câncer de Barretos; LLA = Leucemia Linfocítica Aguda; LLC = Leucemia Linfocítica Crônica; LMA = Leucemia Mieloide Aguda; LMC = Leucemia Mieloide Crônica; LH = Linfoma de Hodgkin; LNH = Linfoma Não Hodgkin; Max = máximo; Min = mínimo; MRD = Matched Related Donor; MUD = Matched Unrelated Donor; MMUD = Mismatched Unrelated Donor.

Os 267 pacientes incluídos contribuíram com um total de 586 amostras de fezes e 877 amostras de sangue. A distribuição do número dessas amostras por centro e por período de coleta está apresentada nas Tabelas 2A e 2B. Observa-se que a maior parte das amostras foi obtida nos períodos iniciais de acompanhamento, com redução progressiva no número de coletas após o D+60.

Tabela 2A: Número de amostras de fezes coletadas de cada centro participante no estudo por ponto de coleta

Centro	D-7	D0	D30	D60	D90	D180	DECHa	Total
FUNFARME	47	48	29	20	15	7	8	174
FAJC	52	50	30	14	8	3	0	157
HCB	13	10	9	6	6	4	2	50
BP	46	50	29	33	23	2	22	205
Total	158	158	97	73	52	16	32	586

Tabela 2B: Número de amostras de sangue coletadas de cada centro participante no estudo por ponto de coleta

FUNFARME	54	60	50	41	30	23	13	271
FAJC	57	58	37	16	12	5	0	185
HCB	15	14	11	8	9	6	2	65
BP	86	94	46	45	36	4	45	356

Total	212	226	144	110	87	38	60	877

BP = Beneficência Portuguesa; D = dias; DECH: Doença do enxerto contra o hospedeiro; D-7 = pré-condicionamento; D0 = dia da infusão das células; DECH = Doença do enxerto contra o hospedeiro aguda; FUNFARME = Fundação Faculdade Regional de Medicina de São José do Rio Preto; FACJ = Fundação Amaral Carvalho de Jau; HCB = Hospital de Câncer de Barretos.

Dentre as amostras de fezes, um total de 491 (84%) já tiveram o DNA extraído. Dessas, 485 (99%) tiveram o sequenciamento do gene 16S rRNA. Em relação às amostras de sangue, 502 (57%) foram analisadas para quantificação de citocinas, e 477 (54%) tiveram os níveis de zonulina determinados.

Em relação aos desfechos clínicos, 40% da coorte desenvolveu DECH aguda durante o período de acompanhamento, enquanto 22% evoluíram a óbito (ver Tabela 3). Entre os desfechos menos frequentemente observados destacam-se a neutropenia febril (18%) e a colite neutropênica (5%).

Tabela 3. Desfechos clínicos dos pacientes inseridos

	FUNFARME (N = 84)	**FACJ (N = 64)**	**HCB (N = 15)**	**BP (N = 104)**	**Total (N = 267)**
Neutropenia Febril	15 (18%)	0 (0%)	10 (67%)	23 (22%)	48 (18%)
Colite Neutropênica	6 (7%)	0 (0%)	3 (20%)	3 (3%)	12 (5%)
Microangiopatia Trombótico associado ao TMO	3 (4%)	2 (3.1%)	0 (0%)	0 (0%)	5 (2%)
DECH	28 (33%)	16 (25%)	5 (36%)	59 (57%)	108 (40%)
Status Último Follow-Up					
Vivo Sem Doença	36 (43%)	52 (81%)	5 (33%)	54 (52%)	147 (55%)
Vivo com Doença	9 (11%)	0 (0%)	0 (0%)	11 (11%)	20 (1%)
Óbito	21 (25%)	12 (19%)	8 (53%)	19 (18%)	60 (22%)
Perda de Follow-up	0 (0%)	0 (0%)	0 (0%)	9 (9%)	9 (3%)

BP = Beneficência Portuguesa; N = número de pacientes; FUNFARME = Fundação Faculdade Regional de Medicina de São José do Rio Preto; FACJ = Fundação Amaral Carvalho de Jau; HCB = Hospital de Câncer de Barretos; TMO = Transplante de medula óssea.

5.2. Manuscritos de Revisão

5.2.1. Manuscrito I – Microbiota Intestinal no Alo-TMO

Journal of *Clinical Medicine*

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Review

Unraveling the Intestinal Microbiota Conundrum in Allogeneic Hematopoietic Stem Cell Transplantation: Fingerprints, Clinical Implications and Future Directions

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Abstract

Intestinal dysbiosis represents a critical determinant of clinical outcomes in patients undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT). Distinct microbiota patterns represent potential prognostic biomarkers and therapeutic targets. However, the exponential growth in microbiota research and analytical complexity has created significant interpretive challenges for clinicians. This review provides a synthesis of current literature examining microbiota fingerprints and their clinical implications. We analyzed key studies evaluating the clinical implications of intestinal microbiota fingerprints in allo-HSCT. Additionally, we examined current therapeutic strategies for microbiota modulation and approaches for translating research findings into clinical practice. We identified three major microbiota fingerprints: (1) decreased intestinal microbiota diversity, (2) reduced abundance of short-chain fatty acid-producing bacteria, and (3) *Enterococcus* domination. These fingerprints are associated with critical clinical outcomes including overall survival, Graft-versus-host disease, transplant-related mortality, and infection-related complications. While fecal microbiota transplantation and dietary interventions appear promising, current studies suffer from limited sample sizes and lack standardized protocols. Despite significant advances in microbiota research, biological, methodological, and logistical challenges continue to hinder its clinical translation. Understanding microbiota fingerprints represents a promising avenue for improving allo-HSCT outcomes. However, successful clinical implementation requires standardized methodologies, mechanistic studies, and multi-center collaborations to translate research into actionable clinical tools.

Keywords: allogeneic hematopoietic stem cell transplantation; Graft-versus-host disease; intestinal microbiota diversity; intestinal dominance; clinical outcomes

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1. Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative therapy for several malignant and non-malignant diseases. However, its effectiveness remains limited due to life-threatening complications, including neutropenic fever, relapse and acute graft versus host disease (aGvHD) [1–6]. Acute GvHD remains the leading cause of non-relapse mortality, affecting up to 70% of patients [1–4]. Relapse remains the primary cause of treatment failure, with incidence reported in up to 40% of cases [6,7]. Similarly, neutropenic fever is highly prevalent, occurring in up to 80% of patients—particularly during the pre-engraftment period [5]. Thus, allo-HSCT is still associated with considerable morbidity. During the allo-HSCT, the intestinal microbiota has emerged as a key player that can shape the development of these poor outcomes. Specific patterns of intestinal microbiota disruption—often referred to as "microbiota fingerprints"—have been linked to these poor outcomes. Consequently, understanding these microbiota fingerprints is increasingly recognized as crucial for predicting outcomes and developing new therapeutic interventions in the transplantation context.

In recent years, there has been a significant increase in studies investigating the role of the intestinal microbiota in allo-HSCT. These studies consistently demonstrate the strong link between intestinal microbiota disruptions and poor clinical outcomes. Nevertheless, the growing volume of data and the complexity of microbiota analysis may pose significant barriers to understanding how these fingerprints impact allo-HSCT patients. Additionally, prior allo-HSCT reviews have focused narrowly on analyzing the relationships of intestinal microbiota with specific diseases and/or complications, failing to provide a comprehensive analysis of clinical implications. Furthermore, most microbiota studies are observational, mechanistically inconsistent and not easily translated into clinical practice. In this review, we aim to clarify this conundrum by summarizing key studies that have evaluated intestinal microbiota fingerprints and their clinical implications for patients undergoing allo-HSCT. After unraveling this conundrum, we will also provide a critical overview of challenges and strategies to: **(1)** modulate the intestinal microbiota, and **(2)** facilitate the integration of intestinal microbiota research into clinical practice. The scientific novelty of this review lies in systematizing current data on microbiota fingerprints and synthesizing them into a comprehensive, clinically meaningful framework that bridges microbiota research with clinical practice.

To conduct this narrative review, an electronic search was performed using PubMed. The search aimed to identify key studies analyzing the relationship between intestinal microbiota and allo-HSCT outcomes. The following keywords were used: "hematopoietic stem cell transplantation", "gut microbiome", "microbiota", "Graft-versus-host disease", "fecal microbiota transplantation", "probiotics", "prebiotics", and "synbiotics". Only key articles providing detailed data regarding intestinal microbiota fingerprints, strategies to modulate intestinal microbiota and allo-HSCT outcomes were included. The search strategy was supplemented by manually reviewing the reference lists of included articles to identify additional relevant studies.

Brief Overview of Human Intestinal Microbiota

The intestinal microbiota comprises a set of commensal microorganisms essential for maintaining human health, including bacteria, fungi, and viruses, which colonize the entire gastrointestinal tract and coexist with the host [8–10]. In healthy adults, the intestinal microbiota is predominantly composed of members from the Bacillota and Bacteroidota phyla, while other microorganisms are present in lower abundance. The intestinal microbiota plays a pivotal role in human physiology, contributing to **(1)** host homeostasis, **(2)** nutrient

metabolism, **(3)** vitamin synthesis, **(4)** immune system modulation, and **(5)** production of metabolites.

The intestinal microbiota has been recognized as a key factor in driving disease development. Intestinal dysbiosis has been linked to inflammatory bowel disease, irritable bowel syndrome, autoimmune diseases and metabolic disorders [8–10]. Intestinal dysbiosis has also been linked to cancer and responses to both immunotherapies and chemotherapies [8,11]. Given the exponential growth of microbiota research and its expanding associations with disease development, severity and treatment responses, it is increasingly important for clinicians to integrate microbiota science into both research and clinical practice. In the following sections, we will highlight the most important patterns of intestinal dysbiosis in the allo-HSCT context and their impacts on clinical outcomes.

2. The Dynamics of Intestinal Microbiota Through the Patient Journey

The patient journey until the allo-HSCT is marked by dynamic and progressive disruptions in the intestinal microbiota. The intestinal microbiota may start to change to a disease-associated layout after the diagnosis of the underlying condition [12,13]. Several studies have demonstrated key features of intestinal dysbiosis even prior to the allo-HSCT [14–19]. The extent of intestinal microbiota disruption during allo-HSCT can be so severe that the re-establishment of homeostasis or eubiosis condition may be markedly delayed, or in some cases, may not be achieved at all [15,17]. Overall, through the patient journey until completing the allo-HSCT procedure, key intestinal microbiota fingerprints may emerge. The most important intestinal microbiota fingerprints identified across studies are: **(1)** decreased intestinal microbiota diversity [16,17,20–27]; **(2)** decreased abundance of SCFA (short-chain fatty acids)-producing bacteria [14,25,28–30]; and **(3)** *Enterococcus* intestinal domination (see Figure 1) [22,24,31–34]. Besides these consistent fingerprints, it is important to note that studies show variable prognostic value for specific bacterial taxa [20,21,35,36]. Multiple studies have identified several taxa associated with either protection or increased risk of poor outcomes. This substantial heterogeneity may result from the lack of methodological standardization across microbiota studies, including differences in sample collection techniques, collection timing, and DNA sequencing methodologies. Due to the lack of consensus and standardization in this area, specific taxa will not be the focus of this review. Instead, we concentrate on the three microbiota fingerprints with the most robust and reproducible evidence linking them to relevant clinical outcomes. These outcomes include overall survival, transplantation-related mortality, aGvHD, infections, and *Clostridioides difficile* colitis (see Figure 2). Given that these intestinal dysbiosis fingerprints have prognostic significance, understanding the factors driving them is critical for improving patient outcomes.

Key factors driving these intestinal dysbiosis fingerprints through the patient journey include chemotherapies, dietary changes and the use of broad-spectrum antibiotics [14,15,20,21,30,36–40]. The relationship between antibiotic exposure and intestinal dysbiosis was demonstrated in a study including 96 patients [20]. When compared to patients (n = 34) without antibiotic exposure, patients (n = 62) receiving any antibiotic in the 3 months prior to allo-HSCT had significantly lower alpha diversity (41.5 $\pm$ 26.54 vs. 61.26 $\pm$ 25.93; p = 0.001) [20]. Similar findings were also reported in a study including 57 patients undergoing allo-HSCT—prior antibiotic use was significantly associated with lower bacterial diversity (p = 0.003) [16]. In this study, other factors associated with intestinal dysbiosis were **(1)** severe underlying hematologic disease ($p < 0.0001$); **(2)** CMV (cytomegalovirus) seropositivity (p = 0.006), **(3)** gastrointestinal or hepatic comorbidities (p = 0.004), and **(4)** recent microbial infection (p = 0.006) [16]. The impact of different conditioning regimens on the intestinal microbiota has also

been evaluated. For example, in a study involving 96 patients undergoing allo-HSCT, those who received myeloablative conditioning exhibited distinct shifts in microbiota composition compared to patients who received reduced-intensity regimens [20]. These findings suggest that several factors contribute to the development of intestinal dysbiosis during allo-HSCT. Nevertheless, whether these factors act individually or synergistically to drive these intestinal dysbiosis fingerprints requires future studies. With a better understanding of the potential drivers of these fingerprints, the following sections will explore the key moments at which these patterns emerge and their potential implications for clinical outcomes.

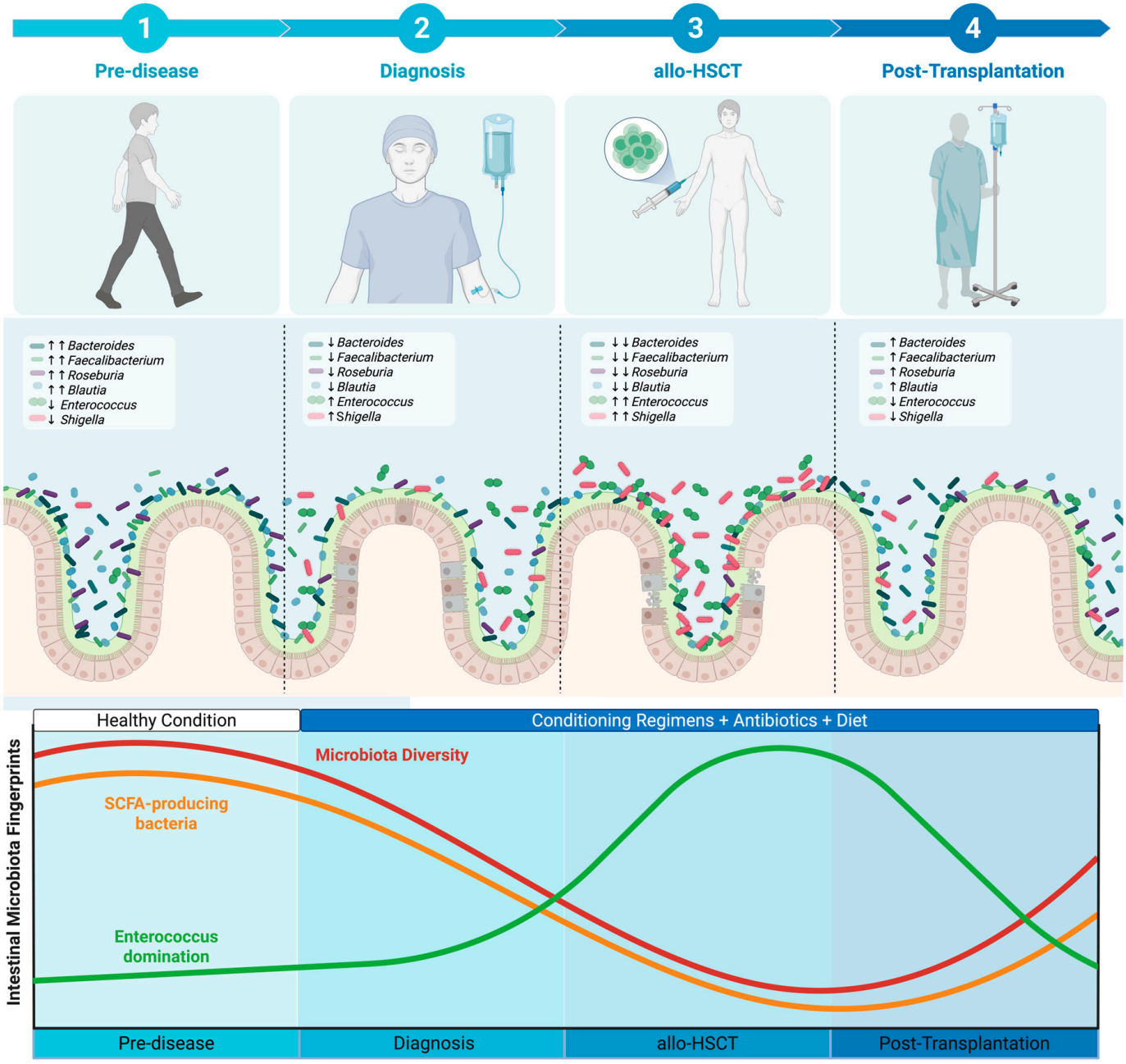


Figure 1. The dynamics of the three intestinal microbiota fingerprints (microbiota diversity, SCFA-producing bacteria and *Enterococcus* intestinal domination) through the allo-HSCT journey. Allo-HSCT = allogeneic hematopoietic stem cell transplantation; SCFA = Short-chain fatty acids; ↑ = increase; ↓ = decrease. Created with BioRender.com, Soares Ferreira Junior A. (2025) https://BioRender.com/8du0b87 (accessed on 19 September 2025).

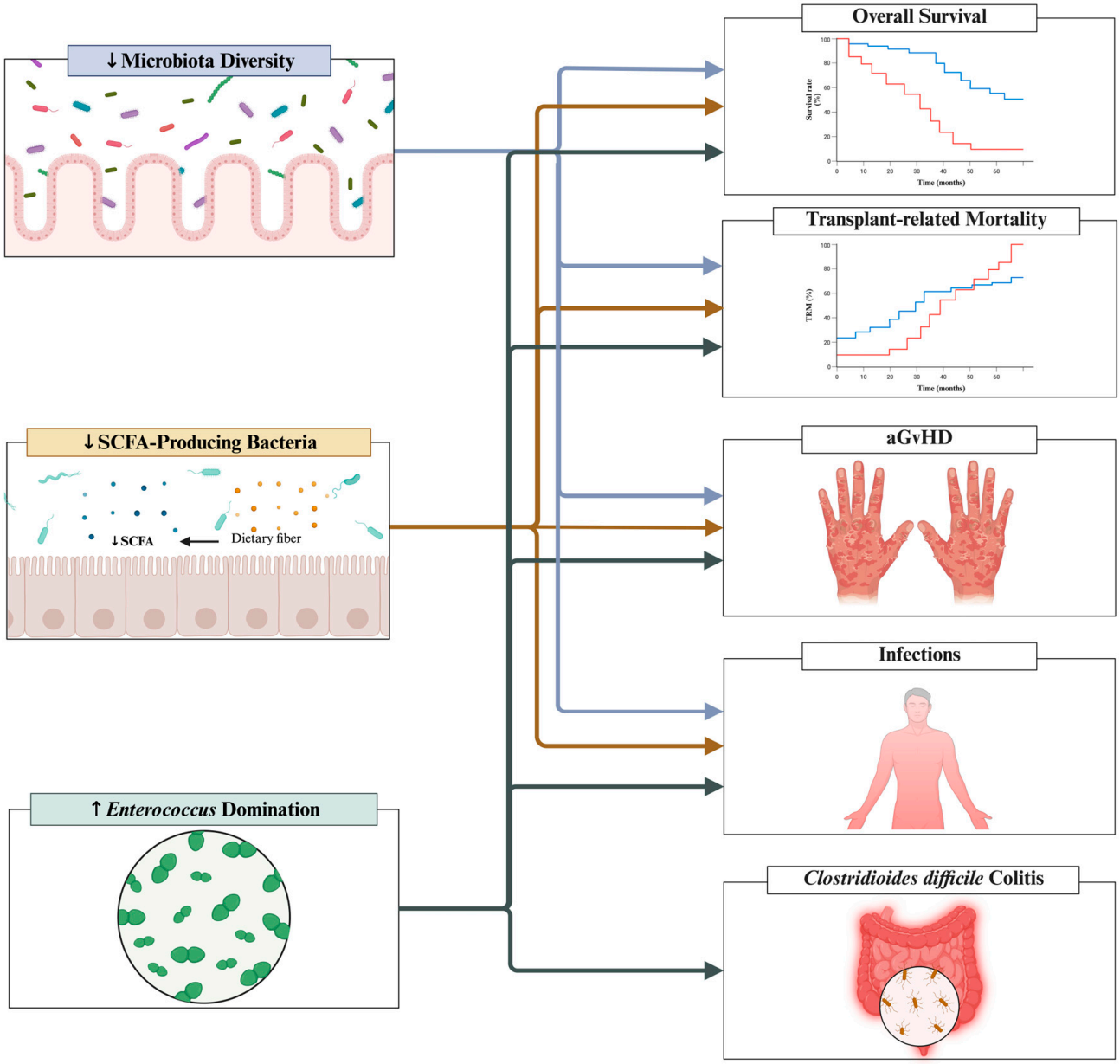


Figure 2. Associations among intestinal microbiota fingerprints (microbiota diversity, SCFA-producing bacteria and *Enterococcus* intestinal domination) and clinical outcomes (overall survival, transplant-related mortality, aGvHD, infections and *Clostridioides difficile* colitis). Red and blue lines in the overall survival and transplant-related mortality figure correspond to patients with high and low intestinal diversity, respectively. aGvHD = acute graft versus host disease; SCFAs = Short-chain fatty acids; ↑ = increase; ↓ = decrease. Created with BioRender.com, Soares Ferreira Junior A. (2025) https://BioRender.com/1f3oh4x (accessed on 19 September 2025).

3. Intestinal Microbiota Fingerprints Prior to Allo-HSCT

In patients undergoing allo-HSCT, there is extensive evidence suggesting that the intestinal microbiota is disrupted even prior to the transplantation [14–19,41]. Prior to allo-HSCT, the most important intestinal dysbiosis fingerprint is decreased intestinal diversity [14,15,19,20,37–41]. Studies have also shown that patients already exhibit a distinct microbiota composition prior to undergoing allo-HSCT (see Supplementary Table S1) [14,15,19,20,37–40]. When compared to a control group (paired HLA [Human Leukocyte Antigen]-matched sibling donors), patients (n = 57) had lower bacterial diversity (p = 0.0002) and different phylogenetic membership (p = 0.001) with increased

relative abundances of facultative anaerobic bacteria (such as *Enterococcaceae* and *Streptococcaceae*) [16]. Furthermore, patients had significantly lower abundance of butyrate-producing bacteria (which produce a key metabolite that helps maintain a healthy gut) compared to healthy volunteers. These bacteria included *Anaerostipes* ($p = 0.036$), *Butyricimonas* ($p = 0.041$), *Coprococcus* ($p < 0.001$), *Faecalibacterium* ($p = 0.014$), and *Lachnospiraceae* ($p < 0.001$) [14]. Similarly, fecal samples from 606 patients showed that the intestinal microbiota was significantly different from healthy adult volunteers and subjects from the Human Microbiome Project [17]. In this study, patients undergoing allo-HSCT had lower intestinal diversity ($p < 0.001$) and a distinct microbiota composition based on enterotype ($p < 0.001$) [17]. Taken together, these and other studies in the literature suggest that intestinal dysbiosis exists even prior to allo-HSCT [14–19]. Given that these intestinal dysbiosis fingerprints have prognostic significance, the next step is to clarify their implication for patient outcomes.

These early intestinal dysbiosis fingerprints are not only present but can significantly shape the course and prognosis of patients undergoing allo-HSCT. These early fingerprints can contribute to the following outcomes: **(1)** overall survival [16,17,20,21]; **(2)** transplantation-related mortality [17]; **(3)** aGvHD [20,21,35,36]; **(4)** and infections [20]. Among the fingerprints, the most evaluated prior to allo-HSCT is the intestinal microbiota diversity—an index that measures the variety (richness) and balance (evenness) of bacteria living in the gastrointestinal tract (see Table 1). Across studies, a consistent finding is that decreased intestinal microbiota diversity prior to allo-HSCT is associated with poor outcomes, such as increased mortality and GvHD [16,17,21,35]. Among the available studies, the most robust in terms of statistical power and sample size was an international multi-center study involving 606 patients. In this study, higher intestinal diversity prior to the transplant was associated with significant reductions in mortality. Specifically, it reduced the risk of death by 59% (HR [Hazard Ratio] 0.41; 95% CI [Confidence Interval] 0.24–0.71) and transplant-related mortality by 56% (HR 0.44; 95% CI 0.22–0.87) [17].

The other fingerprint relates to specific bacterial compositions prior to allo-HSCT that are associated with either protection against or increased risk for poor outcomes (see Supplementary Table S2). For example, higher abundance of *Blautia*, which produces SCFA and promotes gut homeostasis, was associated with lower risk of aGvHD development in two studies [21,35]. Given the prognostic significance of these intestinal dysbiosis fingerprints, future studies should evaluate strategies to incorporate them into risk stratification tools that can be used in clinical practice.

Table 1. Implications of Intestinal Diversity Prior to allo-HSCT.

Outcomes	Author, Year N	Finding
Overall Survival [16,17,21]	Peled 2020 [17] 606	↓ Overall Mortality Higher alpha diversity prior to allo-HSCT was associated with a lower risk of mortality (HR 0.41; 95% CI 0.24–0.71)
	Liu 2017 [16] 57	↓ Overall Mortality Patients with higher phylogenetic diversity had lower overall mortality rates (HR 0.37; 95% CI 0.18–0.77; $p = 0.008$)
	Masetti 2023 [21] β 90	↑ Overall Survival Patients with higher intestinal diversity exhibited a higher probability of overall survival (88.9% ± 5.7% vs. 62.7% ± 8.2%; $p = 0.011$).
Transplantation-related mortality [17]	Peled 2020 [17] 606	↓ Transplant-related mortality Higher alpha diversity prior to allo-HSCT was associated with a lower risk of transplant-related mortality (HR 0.44; 95% CI 0.22–0.87).

Table 1. *Cont.*

Outcomes	Author, Year N	Finding
aGvHD [21]	Masetti 2023 [21] $^{\beta}$ 90	↓ aGvHD The cumulative incidence of grade 2 to 4 aGvHD was significantly lower in the higher diversity group than in the lower diversity group (20.0% ± 6.0% [SE] vs. 44.4% ± 7.4% [SE]; $p = 0.017$). The cumulative incidence of grade 3 to 4 aGvHD was significantly lower in the higher diversity group than in the lower diversity group (2.2% ± 2.2% [SE] vs. 20.0% ± 6.0% [SE]; $p = 0.007$).
	Biagi 2019 [35] 36	The diversity between pre-HSCT samples were greater in individuals who developed intestinal GvHD (0.86 ± 0.15) than in individuals without GvHD (0.72 ± 0.15, $p = 0.001$) and individuals who developed less severe skin GvHD (0.77 ± 0.15, $p = 0.02$).

Allo-HSCT = Allogeneic hematopoieitic stem cell transplantation; CI = Confidence interval; aGvHD = acute graft versus host disease; HR = Hazard ratio; N = number of patients included in this analysis; SE = Standard error; ↓ = decreased; ↑ = increased; $^{\beta}$ = included only pediatric patients.

4. Intestinal Microbiota Fingerprints During Allo-HSCT

The available literature has demonstrated that during allo-HSCT, the already compromised intestinal microbiota undergoes further dysbiosis (see Figure 1) [20–26]. As the intestinal microbiota changes, all three key fingerprints emerge: **(1)** decreased intestinal diversity [20–27,42]; **(2)** decreased abundance of SCFA-producing bacteria [25,28]; and **(3)** intestinal domination by a single taxon [22,24,31–34]. In the following subsections, we will review pivotal studies that have both described these fingerprints and examined their clinical implications.

4.1. Intestinal Diversity and Implications to Clinical Outcomes

During allo-HSCT, the intestinal diversity continues to decline and may not return to baseline levels [17,20–25,27,43]. In a previous study including 96 patients, stool samples were collected at three timepoints: **(1)** baseline (prior to the conditioning regimen), **(2)** D (day) + 10, and **(3)** D + 30 [20]. Compared to baseline, samples collected at both D + 10 and D + 30 showed a significant reduction in intestinal diversity (D + 10: 4.65 ± 1.36 vs. 3.08 ± 1.77; $p < 0.001$; D + 30: 4.65 ± 1.36 vs. 2.62 ± 1.62; $p < 0.001$) [20]. This study also identified a significant reduction in intestinal diversity in samples collected at D + 30 when compared to D + 10 samples (2.62 ± 1.62 vs. 3.08 ± 1.77; $p = 0.020$) [20]. Similar findings were also reported in a study involving 100 patients. Stool samples were collected at four timepoints: **(1)** baseline (prior to the conditioning regimen), **(2)** around the day of stem cell infusion (D − 4 to D0), **(3)** engraftment (D + 4 to D + 28), and **(4)** late post-HSCT (after D + 28) [24]. This study showed a significant reduction in intestinal diversity when comparing baseline samples to those collected around the day of stem cell infusion ($p < 0.05$) and engraftment ($p < 0.01$) [24]. Although intestinal diversity significantly increased in late post-HSCT samples compared to those collected during engraftment ($p < 0.05$), it remained below baseline levels [24]. Taken together, these and other studies demonstrate that intestinal diversity reaches its lowest values within 30 days after the allo-HSCT and gradually increases thereafter [17,20–25,27,43]. However, it often remains below baseline even in samples collected 100 days post-transplant (see Supplementary Table S3). Thus, future studies are needed to explore strategies to preserve and restore intestinal diversity over the allo-HSCT course.

Strategies to preserve and restore the intestinal diversity have become a priority as research has shown it to be a key prognostic factor in patients undergoing allo-HSCT [15,44,45]. In the literature, intestinal diversity during allo-HSCT has been linked to overall survival [17,23,24], aGvHD [20,26,28,41,46], and transplantation-related mortality (see Table 2) [23,24,41]. In most of the studies, lower intestinal diversity in samples collected at the engraftment period has been associated with these poor outcomes [17,20,23,24,28,41,46]. Furthermore, lower intestinal diversity at the time of aGvHD diagnosis has been linked with a severe disease phenotype (see Table 2 for details) [26].

Table 2. Clinical Implications of Intestinal Diversity Over The allo-HSCT.

Outcome	Author, Year N Sample Timing	Finding
Overall Survival [17,23,24]	Peled 2020 [17] 704 At engraftment	↑ Overall Survival Patients were categorized into low- vs. high-diversity groups based on the median value. High diversity at engraftment was associated with a significant improve in overall survival (HR 0.75; 95% CI 0.58–0.96). This association was also identified after multivariable adjustment for age, intensity of the conditioning regimen, graft source and HCT-CI (HR 0.71; 95% CI 0.55–0.92). When considered as a continuous variable, high intestinal diversity was also associated with improved overall survival in both univariate (HR 0.58; 95% CI 0.37–0.91) and multivariate (HR 0.50; 95% CI 0.31–0.80) analysis.
	Taur 2014 [23] 80 At engraftment	↑ Overall Survival Overall survival at 3 years was 36%, 60% and 67% for low, intermediate and high diversity groups (p = 0.19). Patients with low diversity (inverse Simpson < 2) were 3 times more likely to die within the follow-up when compared to those with higher microbial diversity (HR 3.13, 95% CI 1.39–7.98; p = 0.05; adjusted HR 2.56; 95% CI 1.03–7.23; p = 0.42). Low diversity showed a strong effect on mortality after multivariate adjustment for other clinical predictors (transplant related mortality: adjusted hazard ratio, 5.25; p = 0.014).
	Gu 2022 [24] 86 At engraftment	↑ Overall Survival Patients were categorized into low- vs. high- diversity groups based on the median Shannon Index value. When compared to patients with low diversity, patients with high diversity had significantly higher two-year overall survival (83.7% vs. 60.6%; p = 0.026). After adjusting for disease risk, pretransplant comorbidity, and previous chemotherapy, low intestinal diversity was an independent predictor of all-cause death (HR 2.62; 95% CI 1.06–6.49; p = 0.038) in a multivariate analysis.

Table 2. *Cont.*

Outcome	Author, Year N Sample Timing	Finding
Transplantation-related mortality [23,24,41]	Taur 2014 [23] 80 At engraftment	↑ Transplant-related mortality Transplant-related mortality was 9%, 23%, and 53% for high, intermediate and low diversity groups, respectively ($p = 0.03$). Patients with low diversity (inverse Simpson < 2) were 7.5 times more likely to experience transplant-related mortality within the follow-up when compared to those with higher microbial diversity (HR 7.54; 95% CI 2.12–47.88; $p = 0.001$; adjusted hazard ratio, 5.25; 95% CI 1.36–35.07; $p = 0.014$).
	Gu 2022 [24] 86 At engraftment	↑ Transplantation-related Mortality When compared to patients in the high diversity group, patients in the low-diversity group had higher estimated 2-year transplanted related mortality (20.0% vs. 4.7%; $p = 0.04$). After adjusting for pretransplant comorbidity, disease status at the time of allo-HSCT and previous chemotherapy, low intestinal diversity was an independent predictor of transplant-related mortality (HR 4.95; 95% CI 1.03–23.76; $p = 0.046$).
	Galloway-Pena 2019 [41] 44 At engraftment	↓ Transplantation-related Mortality The Shannon diversity index at the time of engraftment was significantly associated with transplantation-related mortality (coefficient = −1.44; $p = 0.02$)
aGvHD [20,23,26,28,41,46]	Jenq 2015 [28] 64 D + 12	↓ GvHD-related mortality Increased intestinal diversity was associated with reduced GvHD-related mortality ($p = 0.005$).
	Mancini 2017 [20] 96 D + 10	↑ aGvHD Decreased intestinal diversity at D + 10 was associated with increased risk of early onset aGvHD (OR 7.833; 95% CI 2.141–28.658; $p = 0.038$).
	Taur 2014 [23] 80 At engraftment	↑ GvHD-related mortality GvHD-related mortality was higher in patients with low diversity ($p = 0.018$).
	Payen 2020 [26] 70 At the onset of GvHD	↑ aGvHD severity Patients with severe aGvHD had significantly lower indexes of alpha diversity: Chao1 ($p = 0.039$) and Simpson ($p = 0.013$)
	Golob 2017 [46] 66 At engraftment Weekly samples from prior to allo-HSCT until D + 100	↑ aGvHD severity Patients with severe aGvHD had a significantly lower alpha diversity index compared to both the control group and patients without severe aGvHD ($p < 0.05$). This finding was statistically significant when analyzing all stool samples collected over the allo-HSCT and when analyzing only samples collected at the engraftment period.
	Galloway-Pena 2019 [41] 44 At engraftment	The Shannon diversity index at the time of engraftment was significantly associated with the incidence of aGvHD ($p = 0.02$)
Infections [23]	Taur 2014 [23] 80 At engraftment	↑ Infection-related mortality Infection related mortality was higher in patients with low diversity ($p = 0.018$).

Allo-HSCT = allogeneic hematopoietic stem cell transplantation; N = number of patients included in the analysis; aGvHD = acute graft versus host disease; ↓ = decreased; ↑ = increased.

4.2. SCFA-Producing Bacteria and Implications to Clinical Outcomes

SCFAs are key microbiota metabolites involved in the mechanisms through which the intestinal microbiota may influence clinical outcomes following allo-HSCT [25,26,28–30,42,47–50].

SCFAs, which include butyrate, propionate and acetate, play important roles in promoting gut homeostasis and regulating the immune system [42,47–49].

During allo-HSCT, there is extensive evidence demonstrating a decrease in SCFA levels and SCFA-producing bacteria (see Figure 1) [25,28,29,42]. In a study with 42 patients, fecal butyrate and propionate were measured at three timepoints: **(1)** Prior to allo-HSCT (baseline), **(2)** D + 7, and **(3)** D + 14 [25]. Compared to baseline, samples collected at D + 7 and D + 14 showed significantly decreased levels of both butyrate and propionate (p-values NR [Not Reported]) [25]. In another study involving 201 patients, stool samples were collected longitudinally at seven timepoints. These included **(1)** Prior to allo-HSCT, **(2)** D0, **(3)** D + 7, **(4)** D + 14, **(5)** D + 21, **(6)** D + 30, and **(7)** D + 90 [30]. This study demonstrated a strong and prolonged suppression of fecal butyrate levels. Significant reductions were observed from prior to allo-HSCT to D0 ($p = 0.01$; r = 0.5) and between prior to allo-HSCT and D + 7 ($p = 0.003$; r = 0.6) [30]. Similarly, in a study of 360 patients, SCFA-producing bacteria were assessed at the time of engraftment [29]. The majority of patients had either a low relative abundance (40.8%) or no detectable (40%) SCFA-producing bacteria, while only 19.2% had a high relative abundance [29]. These findings consistently outline a profound depletion of SCFA and their producers over the allo-HSCT journey, reinforcing their potential role in post-transplantation outcomes.

Indeed, previous studies have demonstrated that decreased levels of SCFAs and their producers contribute to poor allo-HSCT outcomes (see Table 3). Important clinical outcomes modulated by SCFA and their producers are: **(1)** Overall survival [28]; **(2)** GvHD [25,26,28,30,42]; **(3)** Transplantation-related mortality [30]; and **(4)** Viral lower respiratory tract infection [29]. For instance, in a study involving 360 patients, a high abundance of SCFA-producing bacteria at the engraftment period was independently associated with a fivefold decrease in the risk of viral lower respiratory tract infection (HR 0.22; 95% CI 0.04–0.69; $p = 0.06$) [29]. Furthermore, in a study of 64 allo-HSCT recipients, a higher abundance of *Blautia* (a key SCFA-producing genus) was independently associated with lower GvHD-related mortality (HR 0.18; 95% CI 0.05–0.63; $p = 0.007$) and reduced risk of refractory GvHD (HR 0.3; 95% CI 0.14–0.64; $p = 0.002$) [28]. These and other studies in the literature highlight the clinical relevance of preserving SCFA-producing bacteria over the allo-HSCT journey as a key strategy to improve patient outcomes.

Table 3. Implications of SCFA-producing Bacteria and SCFA Levels Over allo-HSCT.

Outcomes	Author, Year N Sample Timing	Finding
Overall Survival [28]	Jenq 2015 [28] 64 D + 12	↑ Overall Survival Increased *Blautia* abundance was strongly associated with improved overall survival ($p < 0.001$).
Transplantation-related Mortality [30]	Meedt, 2022 [30] 201 aGvHD onset//D + 30	↑ Transplant-related Mortality Low BCoAT copy numbers at D + 30/GvHD were significantly associated with increased risk of transplant-related mortality (HR 4.459; 95% CI 1.1018–19.530; $p = 0.047$).

Table 3. *Cont.*

Outcomes	Author, Year N Sample Timing	Finding
aGvHD [25,26,28,30,42]	Jenq 2015 [28] 64 D + 12	↓ GvHD-related mortality By using a taxonomic discovery analysis, increase in the genus *Blautia* was significantly associated with reduced GvHD-related mortality (p = 0.01). By stratifying patients based on *Blautia* median abundance, patients with higher abundance had reduced GvHD-related mortality (p = 0.04). In a multivariable analysis, *Blautia* abundance remained associated with GvHD-related mortality (HR 0.18; 95% CI 0.05–0.63; p = 0.007). ↓ Refractory GvHD Increased *Blautia* abundance was associated with reduced development of acute GvHD that required treatment with systemic corticosteroids or was steroid refractory (p = 0.01). In a multivariable analysis, *Blautia* abundance remained associated with refractory GvHD (HR 0.3; 95% CI 0.14–0.64; p = 0.002). ↓ Liver GvHD Increased *Blautia* abundance was associated with reduced liver GvHD (p = 0.02).
	Payen 2020 [26] 70 aGvHD onset	↑ aGvHD severity When compared to controls (patients undergoing allo-HSCT without GvHD), patients with severe GvHD had a significant depletion of the *Blautia coccoides group* (p = 0.07). Similar findings were found when compared to patients with mild aGvHD (p = 0.036). ↑ aGvHD severity When compared to controls (patients undergoing allo-HSCT without GvHD), patients with severe GvHD had a significant depletion of *Anaerostipes* (p = 0.015). ↑ aGvHD severity When compared to controls (patients undergoing allo-HSCT without GvHD), patients with severe GvHD had a significant depletion of *Faecalibacterium* (p = 0.011). ↑ aGvHD severity When compared to controls (patients undergoing allo-HSCT without GvHD), patients with severe GvHD had a significant depletion of *Lachnoclostridium* (p = 0.019). ↑ GvHD severity When compared to controls (patients undergoing allo-HSCT without GvHD), patients with severe GvHD had significantly lower levels of total SCFAs (12.50 vs. 2.42; p = 0.0003), acetate (8.87 vs. 2.15; p = 0.002), butyrate (1.11 vs. 0.06; p = 0.001), and propionate (2.33 vs. 0.10; p = 0.0009).
	Romick-Rosendale 2018 [25] 42 D + 14	↓ GvHD When compared to patients that developed GvHD, patients without GvHD had significantly higher levels of butyrate (1.77 vs. 0.0550; p = 0.0142), propionate (6.63 vs. 0.208; p = 0.0108) and acetate (39.6 vs. 7.92; p = 0.047) at samples collected at D + 14.

Table 3. *Cont.*

Outcomes	Author, Year N Sample Timing	Finding
aGvHD [25,26,28,30,42]	Meedt, 2022 [30] 201 aGvHD onset//D + 30	↑ GI-GvHD severity Low BCoAT copy numbers at GvHD onset were correlated with GI-GvHD severity (p = 002; r = 0.3). ↑ GI-GvHD Patients with GI-GvHD had lower BCoAT copy numbers than patients with other organs manifestations (0 copies vs. 3.16 × 10^6 copies; p = 0.006; r = 0.3). ↑ GvHD-related Mortality Patients with low BCoAT copy numbers displayed significantly higher GvHD-associated mortality rate than those with high BCoAT concentrations (p = 0.04).
	Artacho 2024 [42] 70 Prior to allo-HSCT and Engraftment	↑ GvHD A significant decrease in acetate levels was detected in patients who developed GvHD (log2FC median = −2.36; p = 0.049).
Infections [29]	Haak 2018 [29] 360 At engraftment	↓ LRTI The incidence of viral LRTI at 180 days was 17.3% and 16.1% for groups in which butyrate-producing bacteria were absent or low, respectively, and 3.2% for the high butyrate-producing group (p = 0.005). Patients with the highest abundance of butyrate-producing bacteria were independently associated with a fivefold decrease in risk of viral LRTI (HR 0.22; 95% CI 0.04–0.69; p = 0.06).

Allo-HSCT = allogeneic hematopoietic stem cell transplantation; BCoAT = Butyryl-CoA/Acetate CoA-Transferase Gene Copy; CI = Confidence interval; D = day; LRTI = Lower respiratory tract infection; GvHD = graft versus host disease; SCFA = Short chain fatty acid; HR = Hazard ratio; N = number of patients included in the analysis; ↓ = decreased; ↑ = increased.

4.3. Intestinal Domination and Implications to Clinical Outcomes

In patients undergoing allo-HSCT, another important microbiota fingerprint is the expansion of a single microbiota genus leading to intestinal domination. Intestinal domination is a frequent fingerprint that can occur in 28 to 65% of patients undergoing allo-HSCT [17,22,24,31–34,51]. Although intestinal domination is a prevalent fingerprint, the specific genus driving these events may vary across studies (see Supplementary Table S5). For example, in a multi-center study including 1325 patients, *Enterococcus* domination occurred in 65% of patients and was the most common genus to dominate the microbiota [32]. Similarly, *Enterococcus* was also the most common genus to dominate the microbiota in a study including 94 patients [34]. Nevertheless, in a study including 98 patients, *Streptococcus* was identified as the most common genus associated with domination events, occurring in 42% of patients [22]. In this study, other genera responsible for intestinal domination in increasing order were: **(1)** *Akkermansia* (28%), **(2)** *Blautia* (28%), **(3)** *Lactobacillus* (28%), **(4)** *Enterococcus* (36%), and **(5)** *Bacteroides* (38%) [22]. Overall, these findings suggest that while the dominant genus may vary across cohorts, *Enterococcus* consistently emerges as a key driver of intestinal domination events [17,22,24,31–34,51].

Enterococcus is not only a key driver of intestinal domination, but it is also the one most likely associated with poor outcomes. Indeed, while previous studies have linked *Enterococcus* domination with poor outcomes, domination by other genera does not appear to carry the same prognostic significance (see Table 4) [17,22,24,31–34,51]. For instance, in a study with 98 patients undergoing allo-HSCT, *Enterococcus* domination was associated

with the following outcomes: bloodstream infection (63% vs. 35%; $p = 0.01$), *Clostridioides difficile* colitis (34% vs. 16%; $p = 0.04$), overall survival ($p = 0.01$), and treatment-related mortality ($p = 0.02$) [22]. In this same study, however, overall survival was not impacted by domination for the following genera: **(1)** *Bacteroides* ($p = 0.08$), **(2)** *Akkermansia* ($p = 0.14$), **(3)** *Blautia* (p-value NR), **(4)** *Lactobacillus* ($p = 0.52$), and **(5)** *Streptococcus* ($p = 0.70$) [22]. Other allo-HSCT studies have also highlighted the implications of *Enterococcus* domination. In a study including 1325 patients, *Enterococcus* domination was an independent risk factor for decreased overall survival (HR 2.06; 95% CI 1.50–2.82; $p < 0.0001$) [32]. *Enterococcus* domination was also associated with a nine-fold increase in the risk of bloodstream infections (HR 9.35; 95% CI 2.43–45.44; $p = 0.001$) in a study including 94 patients [34]. These studies suggest that *Enterococcus* domination is a key fingerprint with prognostic significance during allo-HSCT. Therefore, future studies should explore strategies to modulate *Enterococcus* domination.

Table 4. Implications of Intestinal Domination to Clinical Outcomes.

Outcomes	Autor, Year N Sample Timing	Implication
Overall Survival [22,32]	Messina 2024 [22] 98 Stools were collected once prior to HSCT, weekly until D + 30 and then at days D + 45, D + 90 and D + 180	↓ Overall survival Patients with *Enterococcus* domination had decreased overall survival ($p = 0.01$). Overall survival *Bacteroides* domination at any time point was not significantly associated with overall survival ($p = 0.08$). *Akkermansia* domination at any time point was not significantly associated with overall survival ($p = 0.14$). *Blautia* domination at any time point was not significantly associated with overall survival (p value NR). *Lactobacillus* domination was not significantly associated with overall survival ($p = 0.52$). *Streptococcus* domination was not significantly associated with overall survival ($p = 0.70$).
	Stein-Thoeringer 2019 [32] 1325 Samples were collected in the early post-transplant period (D0 to D + 21)	↓ Overall survival Patients with *Enterococcus* domination in the early-post transplant period had significantly reduced overall survival in univariate analysis (HR 1.97; 95% CI 1.45–2.66; $p < 0.001$). This finding remained significant in a multivariate analysis adjusted for graft source, age, conditioning intensity, gender and underlying disease (HR 2.06; 95% CI 1.50–2.82; $p < 0.0001$).
Transplantation-related Mortality [22]	Messina 2024 [22] 98 Stools were collected once prior to HSCT, weekly until D + 30 and then at days D + 45, D + 90 and D + 180	↑ Treatment-related mortality Patients with *Enterococcus* domination had increased treatment-related mortality ($p = 0.02$).

Table 4. *Cont.*

Outcomes	Autor, Year N Sample Timing	Implication
aGvHD [32]	Stein-Thoeringer 2019 [32] 1325 Samples were collected in the early post-transplant period (D0 to D + 21)	↑ GvHD-related mortality Patients with *Enterococcus* domination in the early-post transplant period had significantly increased GvHD-related mortality in univariate analysis (HR 2.04; 95% CI 1.18–3.52; $p = 0.05$). This finding remaining significant in a multivariate analysis adjusted for graft source, age, conditioning intensity, gender and underlying disease (HR 2.60; 95% CI 1.46–4.62; $p < 0.01$). ↑ GvHD severity (grade 2–4) Patients with *Enterococcus* domination in the early-post transplant period had significantly increased GvHD severity (grade 2–4) in univariate analysis (HR 1.44; 95% CI 1.10–1.88; $p < 0.01$). This finding remained significant in a multivariate analysis adjusted for graft source, age, conditioning intensity, gender and underlying disease (HR 1.32; 95% CI 1.00–1.75; $p < 0.05$).
Infections [22,34]	Messina 2024 [22] 98 Stools were collected once prior to HSCT, weekly until D + 30 and then at days D + 45, D + 90 and D + 180	↑ BSI Patients with *Enterococcus* domination at any time point had increased risk for BSI (63% vs. 35%; $p = 0.01$).
	Taur 2012 [34] 94 Prior to allo-HSCT After allo-HSCT (until D + 35)	↑ BSI Patients with *Enterococcus* domination had a 9-fold increased risk of VRE bacteremia (HR 9.35; 95% CI 2.43–45.44; $p = 0.001$).
	Taur 2012 [34] 94 Prior to allo-HSCT After allo-HSCT (until D + 35)	↑ BSI Patients with Proteobacteria domination had a 5-fold increased risk of Gram-negative bacteremia (HR 5.46; 95% CI 1.03–19.91; $p = 0.047$).
***Clostridioides difficile* colitis** [22]	Messina 2024 [22] 98 Stools were collected once prior to HSCT, weekly until D + 30 and then at days D + 45, D + 90 and D + 180	↑ *Clostridioides difficile* colitis Patients with *Enterococcus* domination at any time point had increased risk for BSI (34% vs. 16%; $p = 0.04$).
Other [22]	Messina 2024 [22] 98 Stools were collected once prior to HSCT, weekly until D + 30 and then at days D + 45, D + 90 and D + 180	↑ Relapse-related mortality Patients with *Enterococcus* domination had increased relapse-related mortality ($p = 0.08$).

Allo-HSCT = allogeneic hematopoietic stem cell transplantation; BSI = Bloodstream infection; CI = Confidence interval; D = day; GvHD = graft versus host disease; N = Number of patients included in this analysis; HR = Hazard ratio; ↓ = decreased; ↑ = increased.

5. Mechanistic Insights Modulating the Relationship Between Intestinal Microbiota and Allo-HSCT

While traditional microbiota studies have focused on taxonomic profiling and remain predominantly associative, the next frontier involves large-scale multiomics analysis to elucidate underlying mechanistic insights [52]. Results from these analyses have revealed that the intestinal microbiota influences host physiology through two primary mechanisms: **(1)** microbiota metabolites, and **(2)** direct receptor binding [53]. Microbiota metabolites,

which range from small to large molecules, include SCFAs, indole, and secondary bile acids. Direct receptor binding between intestinal microbiota and human cells may occur through several mechanisms, including recognition of PAMPs (pathogen-associated molecular patterns) such as LPS (lipopolysaccharides) [53]. Given our special interest in SCFAs, the following paragraph will provide detailed data on how these metabolites may influence host physiology and allo-HSCT outcomes. Detailed information on the other mechanisms can be found in [53].

SCFAs are produced by the microbiota through the metabolism of non-digestible carbohydrates and regulate several metabolic pathways in the gut and distant organs (liver, adipose tissue, and brain) [53]. The main SCFAs include butyrate, propionate, and acetate [10,53]. SCFAs have key functions that influence immune response and intestinal homeostasis. First, SCFAs, especially butyrate, are an essential energy source for intestinal epithelial cells that contribute to maintaining the gut barrier integrity [10,53]. Also, SCFAs support goblet cells via upregulating mucin-related genes and preserve intestinal mucosal barrier [10,53]. Both functions may aid in preventing bacterial translocation [10]. SCFAs have also been linked to the control of the anaerobic environment in the colon through activation of β-oxidation in the mitochondria [53]. Furthermore, they act as a histone deacetylase inhibitor, facilitating the transcription of genes related to immune regulation and providing anti-inflammatory effects, with interleukin (IL)-10 secretion and regulatory T cell differentiation [10]. Taken together, these and other studies in the literature highlight the potential mechanisms by which SCFAs may impact the aforementioned allo-HSCT outcomes (see Section 4.2) [10,50,53,54]. Understanding these mechanistic insights is a key step towards the development of more efficient therapies.

6. Strategies to Modulate the Intestinal Microbiota During Allo-HSCT

Given the prognostic significance of microbiota fingerprints, several studies have explored strategies to modulate the intestinal microbiota during allo-HSCT [32,55,56]. Overall, two major strategies have been investigated: **(1)** fecal microbiota transplantation (FMT), and **(2)** dietary interventions [32,55–59]. These are promising strategies because they can modulate the complex relationship between microbiota and the immune system through mechanisms that control alloreactivity without further compromising the immune system [60]. These strategies have not only demonstrated the ability to restore microbiota diversity and key microbiota metrics, but have also been associated with better clinical outcomes during allo-HSCT [39,55].

FMT is a procedure in which stool from healthy donors (allogeneic FMT) or from the patient prior to dysbiosis (autologous FMT) is administered to restore intestinal microbiota balance [39,55]. In the allo-HSCT setting, FMT is an emerging therapy that has been shown to be feasible [55,60]. However, given the compromised immune system in patients undergoing allo-HSCT, safety concerns remain an important issue [61]. In general, the most common FMT adverse events reported by studies are mild and include diarrhea, bloating, nausea, vomiting, and abdominal pain [56,62–64]. However, severe adverse events may also occur and include sepsis, BSI, colitis, bowel perforation, hepatic encephalopathy and death [56,62–64]. Despite these concerns, studies are showing that severe adverse events, especially infections, are rare in the allo-HSCT setting [61]. Therefore, FMT has been used both as a prophylactic and therapeutic intervention for patients undergoing allo-HSCT [15,55].

Clinical outcomes that have been improved by FMT include: **(1)** GvHD [60,65], **(2)** Drug-resistant bacteria colonization [65–68], **(3)** BSI [68], **(4)** mortality [67], and **(5)** *Clostridioides difficile* colitis [69–71]. For instance, in a study involving 15 adult patients with steroid-refractory or steroid-dependent GvHD that received allogeneic FMT,

66.7% achieved a complete clinical response (resolution of GvHD symptoms) within one month after treatment [60]. Furthermore, patients who responded to the FMT exhibited a significant increase in both intestinal diversity and the abundance of SCFA-producing bacteria [60]. Another study examined 19 adult patients colonized with multidrug-resistant organisms, of whom 8 received FMT [72]. Compared to those who did not receive FMT, patients treated with FMT demonstrated significantly higher 12-month overall survival (70% vs. 36%; $p = 0.044$) and required fewer intensive care admissions (0% vs. 46%; $p = 0.045$) [72]. Although these studies show promising data, significant challenges remain before FMT can become a standardized treatment in the allo-HSCT setting [61,73]. The available literature demonstrates heterogeneity in FMT approaches, including differences in manufacturing process, route of administration, timing, dosing strategies and donor selection. Additionally, significant regulatory challenges persist regarding FMT [73,74]. Regulatory bodies lack consensus on the classification and regulation of FMT, with some countries categorizing it as a drug, while others classify it as a medicinal product or tissue [73,74]. In addition, several countries have yet to establish any regulatory framework for FMT [74]. This lack of harmonization across all aspects of FMT research poses significant barriers to clinical trial development and clinical translation. Although current preliminary findings are encouraging, further large-scale randomized clinical trials employing standardized methodologies are needed.

Dietary interventions represent another promising therapeutic strategy, encompassing **(1)** Prebiotics, **(2)** Probiotics, **(3)** Synbiotics, and **(4)** Route of nutritional support [32,58,59,75–78]. These dietary interventions have been mostly associated with improvement in the following allo-HSCT clinical outcomes: **(1)** GvHD [32,57–59], **(2)** Diarrhea [77], **(3)** Mortality [59,75,77], and **(4)** Mucositis [77]. In a pilot randomized clinical trial including 40 adult patients, 20 patients received daily synbiotics (seven bacterial strains + fructo-oligosaccharides) [58]. When compared to the control group, patients receiving synbiotics had lower rates of severe GvHD (0% vs. 25%; $p = 0.047$) [58]. Similarly, in another study including 44 adult patients, 22 patients received GFO (combination of glutamine, fiber and oligosaccharides) [77]. Patients receiving GFO had a statistically significant reduction in diarrhea duration (3.73 vs. 7.68 days; $p < 0.0001$) and mucositis duration (3.86 vs. 6.00 days; $p < 0.0330$). GFO administration was also associated with higher survival rate at 100 days after allo-HSCT (100% vs. 77.3%; $p = 0.0091$). Nevertheless, it is important to note that most available studies are observational with small sample sizes. Furthermore, studies demonstrate significant heterogeneity across multiple parameters, including substance dosage, administration timing and routes, and strain selection. Therefore, future research employing larger sample sizes and standardized methodologies is warranted to strengthen the evidence base.

7. Challenges in Translating Intestinal Microbiota Research into Allo-HSCT Clinical Practice

Although significant advances have been made in elucidating the role of intestinal microbiota in the allo-HSCT context, microbiota research remains distant from clinical implementation [79,80]. This loss of translation between microbiota research and allo-HSCT clinical practice stems from several factors, including biological, methodological and logistical challenges [79,80].

The biological challenges are fundamentally rooted in the paucity of evidence supporting mechanistic hypotheses that causally link intestinal microbiota alterations to allo-HSCT outcomes [79,80]. Most available literature remains associative and, thus, it remains unclear whether intestinal microbiota changes represent a cause or consequence of clinical outcomes during the allo-HSCT process [79]. For instance, the

presence of the aforementioned fingerprints could be the consequence of several allo-HSCT variables, such as severe underlying disease, multiple hospitalizations, infectious complications and antibiotic use. Methodological challenges arise primarily from the substantial inter-study variability regarding protocols for intestinal microbiota analysis. Studies exhibit substantial variability across multiple parameters, including sample collection techniques, collection timing, DNA sequencing methodologies and bioinformatics pipelines [79]. Furthermore, the effects of confounding variables that may influence the intestinal microbiota are not consistently accounted for, such as dietary patterns, environmental factors and concurrent medications [79]. Additionally, while microbiota research endorses personalized therapeutic strategies, the logistical challenges associated with implementing microbiota profiling and patient-tailored treatment protocols present substantial barriers to clinical translation [79]. Finally, microbiota studies are often single-center studies that include small to modest cohorts, which hampers the generalizability of the findings [79]. In summary, the substantial variability in microbiota research and lack of mechanistic studies, coupled with the inherent complexity of microbiome analyses and inadequate control of confounding variables (diet, antibiotic use, geographic distribution), prevent most clinicians from integrating microbiota research into allo-HSCT clinical practice [79,80].

To overcome the aforementioned challenges, a multifaceted strategy is required (see Figure 3) [79]. The most critical intervention may be the standardization of microbiota research, which is currently advancing through initiatives such as the STORMS checklist ("Strengthening The Organization and Reporting of Microbiome Studies"), FDA (Food and Drug Administration) oversight and consensus guidelines [73,80,81]. This standardization should ideally encompass all phases of microbiota research, spanning from initial test indication to the reporting and clinical interpretation of microbiota findings [80]. Furthermore, while traditional microbiota studies have focused primarily on taxonomic profiling, future multi-omics studies may provide deeper insights [52]. Host-microbiota interactions and their underlying mechanisms could be better understood through these multi-omics studies, which correlate genomic, transcriptomic, proteomic, and metabolomic data. Additionally, interventional studies should comply with FDA regulations when assessing the efficacy and safety of fecal microbiota transplantation products [73]. Equally important is addressing logistical challenges and designing microbiota studies that answer relevant clinical questions and provide outputs applicable to clinical practice [79,80]. To this end, studies should: **(1)** actively engage key stakeholders—including patients, clinicians, scientists and industry partners—in the research process, and **(2)** conduct rigorous sample size estimation to enhance reliability and generalizability [79,80]. Additionally, researchers should prioritize clinical outcomes with direct relevance to clinical practice [79]. Furthermore, researchers should aim to translate complex microbiota findings into accessible clinical tools that assist physicians with patient stratification and prognosis (risk scores). Finally, fostering communication between microbiota scientists and the medical community through targeted educational initiatives and translational grant opportunities will equip physicians with the requisite knowledge to integrate microbiota research into clinical practice [79].

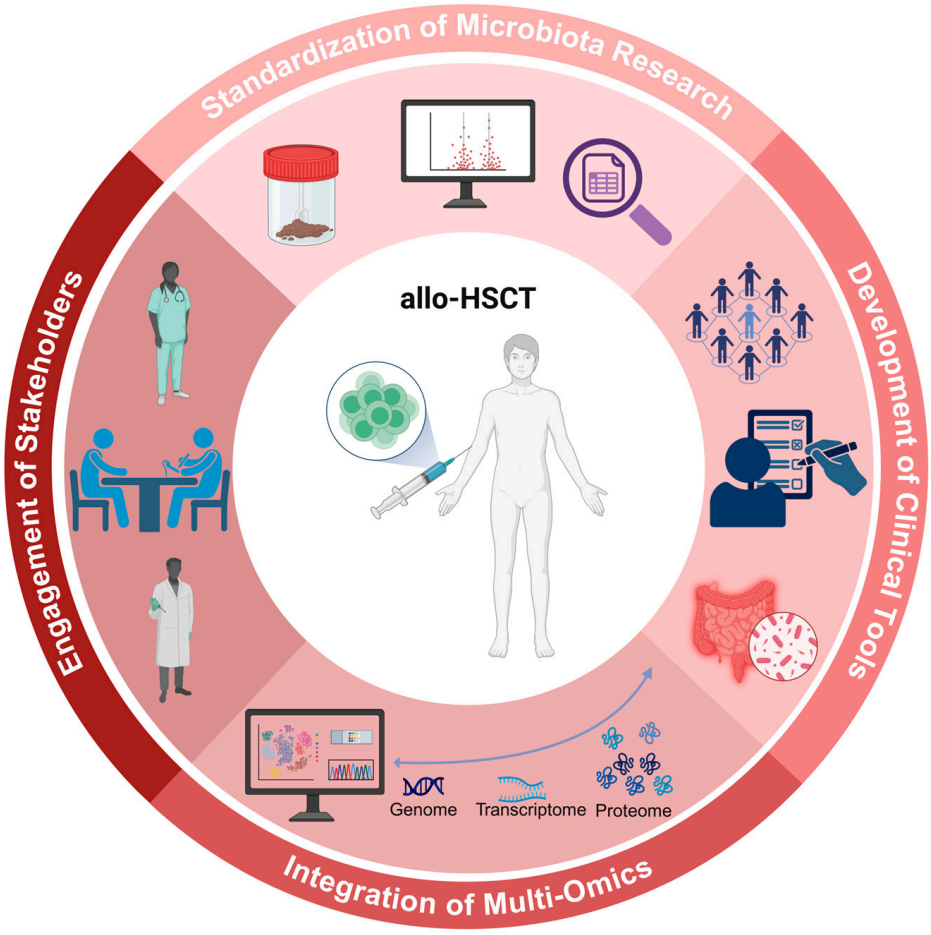


Figure 3. Future directions of intestinal microbiota research in the allo-HSCT context: (1) Standardization of microbiota research, (2) Development of clinical tools, (3) Integration of multi-omics, and (4) Engagement of Stakeholders. Allo-HSCT = allogeneic hematopoietic stem cell transplantation. Created with BioRender.com, Soares Ferreira Junior A. (2025) https://BioRender.com/5d47vvk (accessed on 19 September 2025).

8. Conclusions and Future Directions

This review identified three key intestinal microbiota fingerprints associated with allo-HSCT outcomes: decreased intestinal microbiota diversity, reduced abundance of SCFA-producing bacteria, and *Enterococcus* intestinal domination. Although intestinal microbiota represents a key prognostic factor and therapeutic target in patients undergoing allo-HSCT, further translation of this knowledge into clinical practice is needed. Future large-scale clinical studies with standardized microbiota methodologies and mechanistic evaluation should be designed in collaboration with key stakeholders, including physicians, microbiome scientists, and patients. Such collaborative approaches will enhance study reliability, generalizability, and assessment of outcomes directly relevant to clinical practice.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jcm14196874/s1, Table S1: Key Studies Evaluating the Intestinal Microbiota Prior to allo-HSCT; Table S2: Implications of Intestinal Microbiota Fingerprints Prior to Allo-HSCT; Table S3: Key Studies Evaluating the Dynamics of Intestinal Diversity Over the Allo-HSCT; Table S4: Key Studies Evaluating the Dynamics of SCFA-producing Bacteria and SCFA Levels Over the Allo-HSCT; Table S5: Dynamics of Intestinal Domination Over Allo-HSCT. References [14,16,17,19–25,27–36,41,42] are cited in Supplementary Materials.

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Abbreviations

The following abbreviations are used in this manuscript:

Allo-HSCT	Allogeneic hematopoietic stem cell transplantation
BSI	Bloodstream infection
CI	Confidence interval
CMV	Cytomegalovirus
D	Day
FDA	Food and Drug Administration
FMT	Fecal microbiota transplantation
NR	Not Reported
LRTI	Lower respiratory tract infection
SCFA	Short-chain fatty acid
SE	Standard error
STORMS	Strengthening The Organization and Reporting of Microbiome Studies
GvHD	Graft versus host disease
GFO	Glutamine, fiber and oligosaccharides
HR	Hazard Ratio
HLA	Human Leukocyte Antigen
TRM	Transplantation-related mortality

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Review

Unraveling the Intestinal Microbiota Conundrum in Allogeneic Hematopoietic Stem Cell Transplantation: Fingerprints, Clinical Implications and Future Directions

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Supplementary Data

Supplementary Table S1. Key Studies Evaluating the Intestinal Microbiota Prior to allo-HSCT

Author, year N	Sample Timing Control Group	Intestinal Diversity	Microbiota Composition
Peled 2020[1] 606	First sample obtained from D-30 to D-6. 212 participants in the Human Microbiome Project. 34 healthy adult volunteers.	When compared to the 34 healthy adult volunteers, the 606 patients had lower intestinal diversity ($p < 0.001$). When compared to the 212 participants in the Human Microbiome Project, the 606 patients had lower intestinal diversity ($p < 0.001$) The diversity between the two cohorts of controls were similar ($p = 0.67$).	The composition of stool samples from the 606 patients were distinct from healthy volunteers as assessed according to the fraction of samples that could be categorized to an enterotype ($p < 0.001$).
Liu 2017[2] 57	Samples before conditioning regimen.	When compared to the control group, patients had lower intestinal diversity ($p = 0.0002$)	When compared to the control group, patients had different phylogenetic membership ($p = 0.001$). Patients had increased relative abundancies of facultative

	22 paired HLA-matched sibling donors.		anaerobic bacteria such as *Enterobacteriaceae*, *Lactobacillacea*, *Enterococcaceae* and *Streptococcacea*.
Kusakabe 2020[3] 16	Baseline fecal samples collected prior to conditioning regimens (D-8). 10 healthy volunteers 8 patients undergoing auto-HSCT	By using conventional alpha diversity methods, such as Simpson's index or the Shannon-Wiener index, the intestinal diversity appeared to be similar between allo-HSCT and healthy volunteers.	Among the 16 patients undergoing allo-HSCT, 2 received antimicrobial therapy at the time of the baseline fecal specimen collection. In these two patients, major phyla in the gut microbiota were *Actinobacteria* and *Firmicutes.* For the other patients with antimicrobial exposure, the major phyla were *Bacteroidetes*. Unweighted UniFrac The unweighted uniFrac distance analysis showed significant differences in microbial components between allo-HSCT and healthy controls ($p < 0.001$). Weighted uniFrac The weighted uniFrac distance analysis revealed significant differences in the microbial structure between allo-HSCT and healthy controls ($p < 0.001$) In patients undergoing allo-HSCT, the abundance of genera categorized as butyrate-producing bacteria, such as Anaerostipes, Butyricimonas, Coprococcus, Faecalibacterium and Lachnospiraceae, were significantly lower compared to those in the healthy control group.
Sardzikova 2024[4] 18$^{\beta}$	Samples were collected prior to allo-HSCT 14 healthy children	When compared to healthy children, patients undergoing allo-HSCT had significantly lower alpha diversity ($p < 0.001$).	N/A
Mancini 2017[5] 96	Samples were collected prior	Alpha diversity was significantly reduced in patients who used the following antibiotics in the 3 months	Use of any antibiotics in the 3 months prior to allo-HSCT was associated with a significant decrease in families with the

	to conditioning regimen (T0). N/A	prior to allo-HSCT: 1) any antibiotic (p = 0.001), 2) beta-lactams (p = 0.02), 3) fluoroquinolones (p = 0.05), 4) anti-anerobic therapy* (p = 0.003).	most dominant anaerobic features such as Clostridiaceae (44.15±28.08 vs. 56.44±25.76; p = 0.038), Ruminococcacea (44.13±28.38 vs. 56.47±25.36; p = 0.038) and Veillonellaceae (43.43±28.46 vs. 57.75±24.20; p = 0.016). Use of beta-lactams in the 3 months prior to allo-HSCT was associated with a significant decrease in Peptostreptococcaceae (p = 0.011), Ruminococcaceae (p = 0.016), Veillonelaceae (p = 0.040) and Rickinellaceae (p = 0.018). It was also associated with a significant increase in Enterococcaceae (p = 0.045). Use of fluoroquinolones in the 3 months prior to allo-HSCT was associated with a significant decrease in Clostridiaceae (p = 0.041) Use of anti-anerobic therapy* in the 3 months prior to allo-HSCT was associated with a significant decreased in Clostridiaceae (p = 0.038), Peptostreptococcaceae (p = 0.019), Ruminococcaceae (p = 0.012), Veillonellaceae (p = 0.015) and Rickinellaceae (p = 0.016).
Masetti 2023[6]$^{\beta}$ 90	Samples were collected prior to allo-HSCT. N/A	Higher vs. Lower intestinal diversity prior to allo-HSCT. Median antibiotic exposure was significantly longer in patients with lower intestinal diversity (p = 0.028). Additionally, antibiotic therapy prior to allo-HSCT resulted in a significant reduction in alpha diversity (p = 0.043).	Higher vs. Lower intestinal diversity prior to allo-HSCT. The higher diversity group was characterized by higher relative abundances of the families Oscillospiraceae, Bacteroidaceae, Rikenellaceae, Ruminococcaceae, Prevotellaceae, Coriobacteriaceae, Christensenellaceae, and Tannerellaceae (p < 0.01). The higher diversity group also had higher proportions of *Bacteroides*, *Dorea*, *Parabacteroides*, *Alistipes*, *Collinsella*, *Coprococcus*, *Roseburia*, *Faecalibacterium*, *Blautia*, and members of the *Christensenellaceae* R-7 group, (*Ruminococcus*) torques group, and

			(*Eubacterium*) coprostanoligenes group ($p < 0.05$). The lower diversity group showed an overabundance of Enterococcaceae and Enterobactteriaceae ($p < 0.05$). Additionally, the lower-diversity group was enriched in *Escherichia-Shigella* and *Enterococcus* ($p < 0.05$).
Holler 2014[7] 31	Samples were collected prior to allo-HSCT. 3 donors	N/A	Prior to allo-HSCT vs. Donors At the time of admission, patients showed some loss of dominant commensal strains and an increase in enterococci compared to the donors.
Doki 2017[8] 107	Samples were collected 2 weeks prior to the conditioning regimen. N/A	Low (<2) vs. Intermediate (2-3) vs. High (>3) Intestinal Diversity Among the 107 patients, 18 (16.8%) patients were in the low diversity group, 48 (44.9%) patients were in the intermediate diversity group, and 41 (38.3%) patients were in the high diversity group. Patients in the low diversity cohort were more likely to had antibiotic exposure before the conditioning regimen.	N/A
Meedt 2022[9] 201	Samples were collected at prior to allo-HSCT (D-9 to D-5). 28 healthy donors	N/A	Prior to allo-HSCT vs. Donors There was no difference in copy numbers of fecal Butyryl-CoA/acetate CoA-transferase gene between healthy donors and samples collected prior to allo-HSCT initiation.
Galloway-Pena 2019[10] 44	Samples were collected at the time of conditioning	When compared to healthy donors, patients had significantly lower number of observed OTUs ($p < 0.001$) and Shannon index ($p < 0.001$).	Prior to allo-HSCT vs. Donors When compared to patients, healthy donors showed a high abundance of *Pseudobutyrivibrio* and *Subdoligranulum*. On the

	18 healthy donors		other side, patients showed higher abundance of *Bacteroides*, *Enterobacter*, *Enterococcus* and *Akkermansia*.

Allo-HSCT = allogeneic hematopoietic stem cell transplantation; N = number of patients included in the analysis; N/A = Not applicable; *Anti-anaerobic therapy = included piperacillin-tazobactam, ticarcillin, meropenem, clindamycin, metronidazole and vancomycin; OTU = Operational taxonomic unit; β = included pediatric patients.

Supplementary Table S2. Implications of Intestinal Microbiota Fingerprints Prior to allo-HSCT

Fingerprint	Author, year N	Findings
Enterobacteriaceae abundance >5%[5]	Mancini 2017[5] 96	↑Sepsis Enterobacteriaceae abundance >5% was associated with increased sepsis risk (HR 6.577; 95% CI 2.246-19.255; $p = 0.021$).
Lachnospiraceae abundance ≤10%[5]	Mancini 2017[5] 96	↑All-cause mortality Lachnospiraceae abundance ≤10% was associated with increased all-cause mortality (HR 4.439; 95% CI 2.181-9.035; $p < 0.001$). ↑Infection-related mortality Lachnospiraceae abundance ≤10% was associated with increased infection-related mortality (HR 7.051; 95% CI 2.007-24.778; $p = 0.006$). ↑Non-infection-related mortality Lachnospiraceae abundance ≤10% was associated with increased non-infection-related mortality (HR 21.211; 95% CI 4.949-90.915; $p < 0.001$).
Gram-negative MDR[5]	Mancini 2017[5] 96	↑Non-infection-related mortality Enteric colonization by Gram-negative MDR was associated with increased non-infection-related mortality (HR 10.280; 95% CI 1.536-68.795; $p = 0.048$).
***Blautia* abundance**[6,11]	Masetti 2023[6]β 90	↓aGvHD Higher relative abundance of *Blautia* was protective against subsequent aGvHD development ($p < 0.05$).
	Biagi 2019[11] 36	↓aGvHD When compared to patients who developed GvHD, individuals without aGvHD showed a higher relative abundance of *Blautia* (mainly *Blautia wexlerae*) (8.7% vs. 4.0% [skin GvHD] vs. 1.4% [gut GvHD]; $p = 0.046$). *Blautia* negative association with aGvHD was confirmed by Random Forest analysis ($p = 0.046$)
***Ruminococcus* abundance**[6]	Masetti 2023[6]β 90	↓aGvHD Higher relative abundance of *Ruminococcus* was protective against subsequent aGvHD development ($p < 0.05$).
***Streptococcus* abundance**[6]	Masetti 2023[6]β	↑aGvHD

	90	Higher relative abundance of *Streptococcus* was associated with grade 3 to 4 aGvHD ($p < 0.05$).
***Actinomyces* abundance**[6]	Masetti 2023[6]$^{\beta}$ 90	↑aGvHD Higher relative abundance of *Actinomyces* was associated with grade 3 to 4 aGvHD ($p < 0.05$).
***Lacticaseibacillus* abundance**[6]	Masetti 2023[6]$^{\beta}$ 90	↑aGvHD Higher relative abundance of *Lacticaseibacillus* was associated with grade 3 to 4 aGvHD ($p < 0.05$).
***Rothia* abundance**[6]	Masetti 2023[6]$^{\beta}$ 90	↑aGvHD Higher relative abundance of *Rothia* was associated with grade 3 to 4 aGvHD ($p < 0.05$).
***Firmicutes* abundance**[8]	Doki 2017[8] 107	↑aGvHD Higher abundance of Firmicutes was associated with aGvHD ($p < 0.001$)
***Faecalbacterium* abundance**[8]	Doki 2017[8] 107	↑aGvHD Higher abundance of Faecalbacterium was associated with aGvHD ($p < 0.05$)
***Eubacterium* abundance**[8]	Doki 2017[8] 107	↑aGvHD Higher abundance of *Eubacterium* was associated with aGvHD ($p < 0.05$)
Fusobacterium abundance[11]	Biagi 2019[11] 36	↑aGvHD Individuals who developed a more severe aGvHD had a higher relative abundance of Fusobacterium (0% [no GvHD and skin GvHD] vs. 0.05% [gut GvHD]; $p = 0,01$). Fusobacterium positive association with aGvHD was confirmed by Random Forest analysis ($p = 0.006$)
Allo-HSCT = Allogeneic hematopoieitic stem cell transplantation; CI = Confidence interval; aGvHD = acute graft versus host disease; HR = Hazard ratio; N = number of patients included in this analysis; MDR = Multidrug resistant; ↓ = decreased; ↑ = increased; β = included only pediatric patients;		

Supplementary Table S3. Key Studies Evaluating the Dynamics of Intestinal Diversity Over The Allo-HSCT		
Author, year N	**Samples Timing**	**Intestinal Diversity**
Mancini 2017[5] 96	Samples were collected 1) prior to conditioning regimen; 2) D+10, and 3) D+30	Prior to conditioning regimen vs. D+10 When compared to samples collected prior to the conditioning regimen, samples at D+10 had a significant decrease in alpha diversity metrics: Shannon index: 4.65±1.36 vs. 3.08±1.77; $p < 0.001$. Inverse Simpson index: 0.85±0.13 vs. 0.65±0.27; $p < 0.001$ Observed OTU: 231.61±126.49 vs. 120.23±104.95; $p < 0.0001$ Chao1: 477.49±330.06 vs. 229.51±227.31; $p < 0.0001$ Prior to conditioning regimen vs. D+30

		When compared to samples collected prior to conditioning regimen, samples at D+30 had a significant decrease in alpha diversity metrics: Shannon index: 4.65±1.36 vs. 2.62±1.62; $p < 0.001$. Inverse Simpson index: 0.85±0.13 vs. 0.58±0.26; $p < 0.001$. Observed OTU: 231.61±126.49 vs. 95.77±90.50; $p < 0.001$. Chao1: 477.49±330.06 vs. 180.56; $p < 0.001$. D+10 vs. D+30 When compared to samples collected at D+10, samples at D+30 had a significant decrease in alpha diversity metrics: Shannon index: 3.08±1.77 vs. 2.62±1.62; $p = 0.020$. Inverse Simpson index: 0.65±0.27 vs. 0.58±0.26; $p = 0.041$. Observed OTU: 120.23±104.95 vs. 95.77±90.50; $p = 0.016$. Chao1: 229.51±227.31 vs. 180.56; $p = 0.027$.
Masetti 2023[6]$^{\beta}$ 90	Samples were collected 1) prior to allo-HSCT; and 2) at neutrophil engraftment	Prior to allo-HSCT vs. Neutrophil engraftment The intestinal diversity significantly decreased from prior to allo-HSCT to neutrophil engraftment ($p < 0.0001$).
Messina 2024[12] 98	Samples were collected once prior to HSCT, weekly until D+30 and then at days D+45, D+90 and D+180	Prior vs. After allo-HSCT In patients undergoing allo-HSCT, there is a decline in alpha diversity of the course of therapy, with the lowest values around D+14. Patients demonstrated a subsequent gradual increase in alpha diversity over time, though it does not normalize to baseline alpha diversity even in samples from D+100.
Taur 2014[13] 80	Samples were collected at engraftment	Patients were grouped into 3 levels of microbial diversity based on the inverse Simpson index: high (>4), intermediate (2-4) and low (<2). At the time of stem cell engraftment, high microbial diversity was maintained in 26 (32.5%) patients, intermediate diversity was observed in 20 (25.0%) patient, and low diversity was observed in 34 (42.5%) of patients. Patients who developed acute kidney injury, received any of several antibiotics (intravenous vancomycin, metronidazole, B-lactams), or were diagnosed with *C difficile* infection more frequently fell into the low diversity group.
Gu 2022[14] 100	Samples were collected at 1) D-10, 2) D-4, and 3) every 4 days until D+60 (or hospital discharge). Samples were grouped into the following timepoints 1)	The intestinal diversity decreased to the lowest at approximately D+12 after allo-HSCT and then increased over time. Baseline vs. Pre-HSCT When compared to baseline samples, pre-HSCT samples had a significant decrease in intestinal diversity ($p < 0.05$).

	Baseline (Prior to conditioning); 2) Pre-HSCT (D-4 to D0); 3) Engraftment (D+4 to D+28); and 4) Late Post-HSCT (after D+28)	Baseline vs. Engraftment When compared to baseline samples, samples collected at engraftment had a significant decrease in intestinal diversity ($p < 0.01$). Pre-HSCT vs. Engraftment When compared to pre-HSCT samples, samples collected at engraftment had a significant decrease in intestinal diversity ($p < 0.05$). Engraftment vs. Late Post-HSCT When compared to samples collected at engraftment, samples from late post-HSCT had a significant increase in intestinal diversity ($p < 0.05$)
Romick-Rosendale 2018[15] 42	Samples were collected at prior to allo-HSCT, D0, D+7, and D+14.	Prior to allo-HSCT vs. D+14 When compared to pre-HSCT samples, samples collected at D+14 had a significant decrease in intestinal diversity ($p = 0.0028$)
Sardzikova 2024[4] 18[β]	Samples were collected t prior to allo-HSCT, D+7 and D+28.	Prior to allo-HSCT vs. D+7 In patients who developed febrile neutropenia, intestinal diversity was significantly decreased in samples collected on D+7 compared to pre-HSCT samples ($p = 0.016$).
Artacho 2024[16] 70	Samples were collected at prior to allo-HSCT (4.49 ± 4.24 days before) and engraftment (14.01 ± 1.15 days after)	Prior to allo-HSCT vs. Engraftment Compared to pre–allo-HSCT samples, samples collected at engraftment exhibited a significantly different microbial composition (PERMANOVA $p = 0.002$) and a marked decrease in alpha diversity ($p < 0.0001$). The top significantly different genera between pre allo-HSCT samples and engraftment samples included *Blautia, Clostridium_IV, Roseburia, Oscillibacter, Faecalibacterium, Ruminococcus, Anaerostipes, Intestinimonas, Clostridium_XIVb, Anaerotruncus, Eggerthella, Pseudoflavonifractor, Romboutsia, Intestinibacter, Staphylococcus* and *Lachnospiraceae_UC.* In depth sequencing identified an expansion of three staphylococci species: *S. aureus* ($p < 0.01$), *S. epidermidis* ($p < 0.0001$) and *S. simulans* ($p < 0.0001$).
Allo-HSCT = allogeneic hematopoietic stem cell transplantation; D = day; N = number of patients included in the analysis; OTU = Operational taxonomic unit; β = included pediatric patients.		

Supplementary Table S4. Key Studies Evaluating the Dynamics of SCFA-producing Bacteria and SCFA Levels Over the Allo-HSCT		
Author, year **N**	**Samples Timing** **Bacteria** **SCFA**	**Butyrate-producing Bacteria**
Jenq 2015[17] 64	D+12 *Blautia* N/A	Factors associated with loss of *Blautia* were 1) treatment with antibiotics that inhibit anaerobic bacteria ($p = 0.01$); and 2) total parenteral nutrition for longer durations ($p = 0.01$).
Romick-Rosendale 2018[15] 42	Samples were collected at prior to allo-HSCT, D0, D+7, and D+14. N/A Fecal butyrate and propionate	Prior to allo-HSCT vs. D+7 Fecal butyrate and propionate levels were significantly decreased at D+7 when compared to baseline (values NR). Prior to allo-HSCT vs. D14 Fecal butyrate and propionate levels were significantly decreased at D+14 when compared to baseline (values NR).
Meedt 2022[9] 201	Samples were collected at prior to allo-HSCT, D0, D+7, D+14, D+21, D+30, and D+90 Fecal Butyryl-CoA/acetate CoA-transferase gene copy number	Prior to allo-HSCT vs. D0 Levels were significantly decreased at D0 when compared to baseline ($p = 0.01$; r = 0.5). Prior to allo-HSCT vs. D+7 Levels were significantly decreased at D+7 when compared to baseline ($p = 0.003$; r=0.6).
Haak 2018[18] 360	At engraftment SCFA-producing bacteria Fecal butyrate, propionate and acetate	At engraftment, 40% of patients had no detectable butyrate-producing bacteria. A high relative abundance of these bacteria was observed in only 19.2% of patients, while 40.8% had a low relative abundance.
Galloway-Pena 2019[10] 44	After HSCT N/A Fecal butyrate	Higher fecal butyrate level was associated with higher Shannon diversity index (r = 0.55; $p < 0.01$) and number of observed OTUs (r = 0.67; $p < 0.01$).

		Patients' samples with low levels of butyrate after HSCT was enriched for *Enterococcus* and *Lactobacillus*.
Artacho 2024[16] 70	Samples were collected at prior to allo-HSCT (4.49 ± 4.24 days before) and engraftment (14.01 ± 1.15 days after) N/A Fecal butyrate, propionate and acetate	Compared to pre–allo-HSCT samples, samples collected at engraftment exhibited a significantly decrease in butyrate ($p < 0.0001$), propionate ($p < 0.01$) and acetate ($p < 0.0001$).
Allo-HSCT = allogeneic hematopoietic stem cell transplantation; D = day; N = number of patients included in the analysis; N/A = Not applicable; NR = Not Reported; OTU = Operational taxonomic unit; SCFA = Short chain fatty-acid;		

Supplementary Table S5. Dynamics of Intestinal Domination Over Allo-HSCT

Intestinal Domination	Autor, year N	Sample Timing	Intestinal Domination Dynamics
Any genus[14]	Gu 2022[14] 100	Samples were collected at 1) D-10, 2) D-4, and 3) every 4 days until D+60 (or hospital discharge). Samples were grouped into the following timepoints 1) Baseline (Prior to conditioning); 2) Pre-HSCT (D-4 to D0); 3) Engraftment (D+4 to D+28); and 4) Late Post-HSCT (after D+28)	The prevalence of intestinal domination by a certain bacterial genus was about 80% of all patients before the start of conditioning. The incidence of intestinal domination increased to approximately 95% of all patients during the engraftment period and then began to decrease.
Enterococcus[12,19–21]	Messina 2024[12] 98	Prior to allo-HSCT	2 patients (2.0%) already had *Enterococcus* domination prior to allo-HSCT
		Prior to HSCT (D-30 to D0) Weekly until D+30 At days D+45, D+90; D+180 and annually post-transplant.	36% (n=35/98) of patients undergoing allo-HSCT had *Enterococcus* domination in at least one sample.

			The median duration between transplant date and the first stool sample was 22 days (IQR, 6.75–84.25).
	Fujimoto 2024[19]β 46	Prior to allo-HSCT Weekly until D+98 or hospital discharge	Among the 317 stool samples, *Enterococcus* domination was presented in 89 (28%). Among the 46 patients, Enterococcus domination was observed in 30 (65.2%). Stool cultured was performed from samples with *Enterococcus* domination and 30 enterococci were isolated: 11 strains of *Enterococcus faecalis* and 19 strains of *Enterococcus faecium.*
	Stein-Thoeringer 2019[20] 1325	Prior to allo-HSCT	Among 1059 samples collected prior to allo-HSCT (between D-30 and D-6), 60 (5.7%) had *Enterococcus faecium* domination.
		From D-20 to D+24	*Enterococcus* domination occurred in up to 65% of patients after allo-HSCT. *Enterococcus faecium* was the dominant species in most patients across all centers. *Enterococcus* genus was the most commonly observed to dominate the microbiota in patients from 4 different centers.

		From D0 to D+21	Among 705 patients, *Enterococcus* domination occurred 28.8% (N=203).
		At the time of engraftment (±D+24)	Samples with Enterococcus domination were associated with a loss of *Clostridia spp* (butyrate-producing bacteria).
	Taur 2012[21] 94	Prior to allo-HSCT After allo-HSCT (until D+35)	*Enterococcus* was the most frequent dominating genus, occurring in 40.4% (n=38/94) patients.
	Chhabra 2023[22] 38	Prior to allo-HSCT After allo-HSCT (until D+21)	*Enterococcus* domination was observed in approximately 30% of patients.
Streptococcus[12,21]	Messina 2024[12] 98	Prior to allo-HSCT (D-30 to D0) Weekly until D+30 At days D+45, D+90; D+180 Annually post-transplant.	*Streptococcus* was the most identified genus responsible for intestinal domination at any timepoint (42% of patients).
	Taur 2012[21] 94	Prior to allo-HSCT After allo-HSCT (until D+35)	*Streptococcus* was the second most frequent dominating genus, occurring in 37.2% (n=35/94) patients.
Bacteroides[12]	Messina 2024[12] 98	Stools were collected once prior to HSCT (D-30 to D0), weekly until D+30 and then at days D+45, D+90; D+180 and annually post-transplant.	38% of patients undergoing allo-HSCT had *Bacteroides* domination in at least one sample.[Δ]
Akkermansia[12]	Messina 2024[12] 98	Stools were collected once prior to HSCT (D-30 to D0), weekly until D+30 and then at days D+45, D+90; D+180 and annually post-transplant.	28% of patients undergoing allo-HSCT had *Akkermansia* domination in at least one sample.[Δ]
Blautia[12]	Messina 2024[12] 98	Stools were collected once prior to HSCT	28% of patients undergoing allo-HSCT had

		(D-30 to D0), weekly until D+30 and then at days D+45, D+90; D+180 and annually post-transplant.	*Blautia* domination in at least one sample.[Δ]
Lactobacillus[12]	Messina 2024[12] 98	Stools were collected once prior to HSCT (D-30 to D0), weekly until D+30 and then at days D+45, D+90; D+180 and annually post-transplant.	28% of patients undergoing allo-HSCT had *Lactobacillus* domination in at least one sample.[Δ]
IQR = Interquartile range; N = Number of patients undergoing allo-HSCT; D = day; Δ = estimated values obtained from a graph; β = intestinal domination defined as relative abundance ≥25%			

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5.2.2. Manuscrito II – Permeabilidade Intestinal no Alo-TM

HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Review article

Intestinal permeability in allogeneic hematopoietic stem cell transplantation: A comprehensive review of measurement methods, clinical determinants and prognostic significance

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ABSTRACT

The role of intestinal permeability in patients undergoing allogeneic hematopoietic stem cell transplantation remains unclear. This narrative review aimed to critically analyze the available evidence regarding the dynamics, predictors, and prognostic significance of intestinal permeability in the allogeneic hematopoietic stem cell transplantation setting. PubMed was searched from inception to December 2025 to identify relevant studies. Inclusion criteria comprised articles that used any methodology to assess intestinal permeability in the allogeneic hematopoietic stem cell transplantation setting. Animal studies and non-English publications were excluded. Twenty-one studies were identified involving 1,130 patients that used 22 distinct methodologies, encompassing both probe-based and biomarker-based approaches. While most probe-based studies demonstrated significant temporal changes, biomarker-based methodologies yielded conflicting and non-significant findings. Consistent predictors of increased intestinal permeability across the transplantation course included the conditioning regimen, dietary interventions, intestinal microbiota, and immune response. Regarding the prognostic significance, studies reported heterogeneous and often conflicting results across most evaluated clinical outcomes. Although significant advances have been made in understanding the role of intestinal permeability in the allogeneic hematopoietic stem cell transplantation setting, substantial knowledge gaps remain. Future studies should use multiple complementary methodologies simultaneously to capture the different components of the intestinal barrier.

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Introduction

In patients undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT), intestinal permeability has emerged as a potential prognostic factor to assist in risk stratification and clinical management [1,2]. In the allo-HSCT setting, alterations in intestinal permeability have been investigated as a key underlying mechanism in the complex interplay between host physiology and the intestinal microbiota. This complex interplay has become the focus of extensive research over recent years [3]. Specific patterns of intestinal microbiota disruption, commonly referred to as features of intestinal dysbiosis, have been associated with adverse allo-HSCT-related outcomes [4–6]. For instance, reduced intestinal microbiota diversity has been linked to decreased overall survival, whereas intestinal microbiota domination by a single bacterial taxon has been associated with the development of acute graft-versus-host disease (aGVHD) [4]. In addition, other intestinal microbiota fingerprints have been linked to clinically relevant outcomes, including transplant-related mortality, infectious complications, and *Clostridioides difficile* colitis [4].

Improving allo-HSCT prognosis requires a detailed understanding of the mechanisms underlying the interaction between the intestinal microbiota and the host. Increased intestinal permeability has emerged as a central pathophysiological process modulating this interaction [1,2]. This increase in intestinal permeability may result from injury to the gastrointestinal barrier induced by several factors, including conditioning regimens, antibiotic exposure, and infections. Such injury facilitates the translocation of luminal antigens, dietary components, and microorganisms to the lamina propria and systemic circulation, thereby contributing to the development of allo-HSCT–related outcomes. Although several studies have evaluated the potential prognostic significance of intestinal permeability in the allo-HSCT setting, they are often limited by substantial heterogeneity in the components assessed and in the methodologies used to evaluate intestinal barrier function [7–13]. Moreover, rather than focusing on the dynamics and prognostic implications of intestinal permeability, previous investigations have primarily addressed strategies aimed at preserving barrier integrity [14]. Therefore, there is a clear need for future studies to critically analyze and integrate the current evidence regarding intestinal permeability in this clinical context.

This narrative review aims to provide a comprehensive and critical overview of: 1) the anatomy and physiology of the normal gastrointestinal mucosal barrier; 2) the methodologies used to assess intestinal permeability in the allo-HSCT setting; and 3) the dynamics, modulators, and prognostic significance of intestinal permeability in patients undergoing allo-HSCT.

Methods

A comprehensive literature search was conducted in PubMed from database inception through December 2025 to identify studies investigating intestinal permeability following allogeneic allo-HSCT. The search strategy was structured around two primary concepts: (1) hematopoietic stem cell transplantation and (2) intestinal permeability (Supplementary Search Strategy). The screening process was conducted by a single reviewer (ASFJ). Inclusion criteria were English-language studies that evaluated any method of assessing intestinal permeability (including both probe-based and biomarker-based approaches) in patients undergoing allo-HSCT. Animal studies were excluded. The search was complemented by manual screening of reference lists from included articles to identify additional relevant studies. Finally, the reference list of a prior review on intestinal permeability in the allo-HSCT setting was also reviewed [14].

Data extraction was conducted by two independent reviewers using template tables. Any disagreements were resolved through discussion between these two reviewers. Among the variables extracted were author and year of publication, intestinal permeability methodologies, dynamics of intestinal permeability throughout allo-HSCT, factors associated with intestinal permeability, and associations between intestinal permeability and clinical outcomes. Although this is not a systematic review, the PRISMA checklist was used as a template, and applicable items were incorporated to improve the transparency of this paper.

Results and discussion

Included studies

The search strategy identified a total of 107 records. Following title and abstract screening, 79 studies were excluded. Consequently, 28 studies proceeded to full-text review. An additional 15 studies were excluded after full-text review, most commonly because they did not assess intestinal permeability or were based on animal models. Manual reference searching yielded eight additional articles (Supplementary Table 1) [2,7–11,15]. Therefore, 21 studies comprising 1,130 patients met the eligibility criteria and were included in this review (Supplementary Table 2). The majority of studies (n = 14; 66.7%) included only adult patients, while six studies (28.6%) included both pediatric and adult populations; none evaluated pediatric patients exclusively. The three main findings of this narrative review are summarized in Figure 1.

The healthy gastrointestinal barrier

The gastrointestinal barrier is a complex physical structure that separates the internal environment of the body from the intestinal lumen and exhibits selective permeability, allowing the controlled transport of compounds and microorganisms beneficial to the host while preventing the entry of harmful substances, pathogens, and food antigens [16]. It is primarily composed of three layers: (1) the mucus layer, including inner and outer sublayers that harbor the intestinal microbiota; (2) the intestinal epithelium; and (3) the lamina propria, which contains immune cells [16]. The mucus layer is formed mainly by mucins and commensal microorganisms, including bacteria, fungi, viruses, and parasites [16]. These commensal microorganisms play key roles in maintaining host

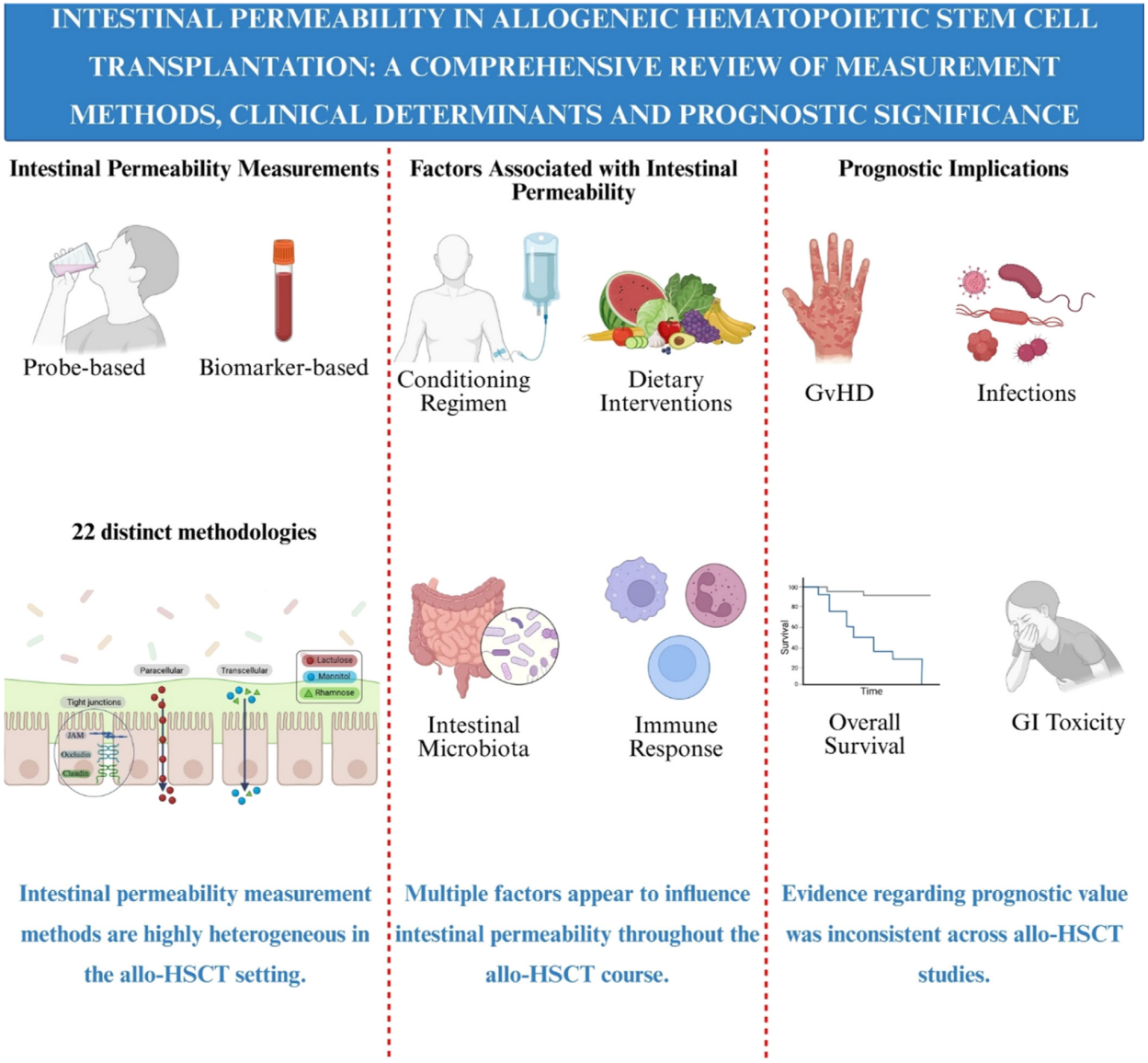


Fig. 1 – Key findings on intestinal permeability in the allo-HSCT setting. Allo-HSCT: Allogeneic hematopoietic stem cell transplantation;GVHD: Graft versus host disease; GI: Gastrointestinal.

homeostasis, contributing to digestion, vitamin biosynthesis, metabolite production, and immune system development (helping to distinguish self from non-self antigens) [4,16]. The second layer, the intestinal epithelium, consists of a single layer of specialized cells, including enterocytes, goblet cells, Paneth cells, enteroendocrine cells, and microfold cells [16]. Finally, the third layer, the lamina propria, comprises components of the enteric immune system, including macrophages and dendritic cells.

Microorganisms, metabolites, antigens, and dietary particles present in the intestinal lumen may traverse the gastrointestinal barrier through multiple routes, a process that underlies the concept of intestinal permeability [16,17]. This concept is multifaceted and encompasses alterations in both transcellular pathways, mediated by membrane transporters and endocytic mechanisms, and paracellular routes, which are regulated by tight junctions [16,17]. Tight junctions are protein complexes that regulate paracellular permeability by joining adjacent epithelial cells [16,17]. These tight junction complexes are composed of transmembrane proteins, such as occludin and claudins, and peripheral membrane adaptor proteins, including members of the zonula occludens family, which anchor the junctional complex to the actin cytoskeleton [16,17]. Classically, the paracellular route has been the pathway most closely associated with disease development [17]. However, accumulating evidence in recent years indicates that the transcellular route also plays a critical role in pathogenesis [17]. In the allo-HSCT setting, both transcellular and paracellular routes may be altered, and specific methodological approaches are available to assess each of these pathways [17].

Intestinal permeability measurement in the allogeneic hematopoietic stem cell transplantation setting

In the allo-HSCT setting, numerous studies have investigated methodologies to quantify intestinal permeability. Overall, approaches to assess intestinal permeability can be broadly categorized into two principal types: 1) Probe-based methods, and 2) Biomarker-based methods. The most commonly used methodologies in the allo-HSCT setting include: 1) Citrulline (eight studies with 342 patients) [7–13]; 2) Lactulose:Rhamnose Ratio (L:R - five studies with 186 patients) [3,10,11,13,15]; 3) Lactulose:Mannitol Ratio (L:M - four studies with 165 patients) [1,12,18,19]; 4) Intestinal fatty acid–binding protein (I-FABP: four studies with 355 patients) [7,8,20,21]; 5) Lipopolysaccharide-binding protein (LBP: four studies with 302 patients) [2,3,13,20]; 6) Chromium-51–labeled ethylenediaminetetraacetic acid absorption (51Cr-EDTA: three studies with 80 patients) [22–24]; 7) Xylose:3-O-methyl-D-glucose (X:O Ratio: three studies with 74 patients) [11,13,15]; 8) Rhamnose:3-O-methyl-D-glucose (R:O Ratio: three studies with 74 patients) [11,13,15]; and 9) Regenerating islet-derived protein 3 alpha (REG3α: two studies with 175 patients). Other methodologies used in one study each included suppression of tumorigenicity (ST2) [3], occludin [3], claudin1 [3], intestinal alkaline phosphatase (IAP) [3], zonulin [1], and calprotectin [1].

Each of these methodologies has specific advantages and limitations (Supplementary Table 3). Overall, probe-based approaches rely on measuring the urinary excretion of orally administered compounds that are poorly absorbed within the gastrointestinal tract [25,26]. These methodologies include: 1) L:M Ratio, 2) L:R Ratio, and 3) the 51-CrEDTA absorption test. Although these are well-established methods that provide a direct functional assessment of intestinal permeability, they typically require active patient participation and may be poorly tolerated in individuals with extensive gastrointestinal tract injury (a common feature in the allo-HSCT setting) [1,3,12,18,27]. Furthermore, when probe-based methodologies are expressed as ratios, false-negative results may occur if absorption of both the paracellular and transcellular probes is increased proportionally, which may give the impression that intestinal permeability is normal [28]. Finally, the permeability route (paracellular vs. transcellular) of many of these probes remains unclear, thereby limiting a comprehensive understanding of the underlying mechanisms of intestinal permeability [17].

In contrast, biomarker-based methodologies represent surrogate measures of intestinal permeability and can be assessed using serum or stool samples. These approaches include citrulline, zonulin, I-FABP, LBP, calprotectin, β-defensin-2, sCD14, myosin light chain kinase 210 isoform (MLCK210), REG3α, ST2, IAP, and claudin-3. A major advantage of biomarker-based methods is their feasibility in clinical practice, as these tests are easy to perform and do not require active patient participation [26,29]. However, further validation and standardization are needed, given the variability in sensitivity and specificity reported across different clinical settings [30,31].

This heterogeneity in intestinal permeability methodologies in the allo-HSCT setting becomes problematic for several reasons, especially considering that none of these studies have properly validated these methodologies against the gold standard methodology (Ussing chambers) [17]. First, these assays assess distinct components of the intestinal barrier and therefore represent different biological concepts [1,8]. For instance, citrulline is a surrogate marker of enterocyte mass, zonulin is a tight junction modulator, REG3α is a surrogate of Paneth cell injury, and I-FABP and I-BABP are surrogates of enterocyte turnover [1,8]. Thus, although they are broadly categorized as intestinal permeability markers, each has distinct regulatory mechanisms and temporal dynamics over the course of allo-HSCT. Second, the gastrointestinal tract is a long and anatomically heterogeneous system, with different barrier characteristics along its length (e.g., small intestine vs. colon). Accordingly, some assays are more specific to certain regions: while I-FABP is expressed in enterocytes of both the small and large intestine, I-BABP is exclusively expressed in the jejunum and ileum [8]. Finally, distinct mechanisms may underlie the increased intestinal permeability at different time points over the course of allo-HSCT. In a study evaluating intestinal permeability in mice undergoing allo-HSCT, different mechanisms were identified throughout the transplant course [32]. Whereas increased intestinal permeability in the early post-transplant period was primarily related to gross epithelial damage, the increase observed during graft-versus-host disease (GvHD) propagation was driven by myosin light chain kinase 210 isoform (MLCK210)-dependent alterations in tight junction permeability [32]. This is further supported by several studies demonstrating conflicting correlations among

some of these intestinal permeability surrogates (Supplementary Table 4). Taken together, these findings highlight the need for time-specific selection and validation of intestinal permeability assessment methodologies throughout the allo-HSCT course. Understanding how intestinal permeability evolves over time during allo-HSCT is essential to optimize methodology selection and to interpret results within an appropriate biological and clinical context. In the following section, the current evidence demonstrating intestinal permeability dynamics over the course of allo-HSCT will be further explored.

Intestinal permeability dynamics over the allo-HSCT

The dynamics of intestinal permeability were evaluated in 18 studies including 834 patients using a total of 21 different methodologies (Table 1). Probe-based methodologies were used in 12 studies involving 425 patients [1,3,10–13,15,18,19,22–24]. Furthermore biomarker-based methodologies were used in 13 studies, but evaluated in a total of 657 patients [1–3,7–13,20,32]. Across methodologies, studies consistently point toward increased intestinal permeability in the allo-HSCT setting (Supplementary Table 5).

Overall, studies using probe-based methodologies indicate that both transcellular and paracellular permeability increase over the first month following allo-HSCT. Interestingly, patients undergoing allo-HSCT already exhibited increased intestinal permeability prior to the procedure when compared with healthy controls (two studies) [1,18]. This impairment was further exacerbated over time, with a trend toward higher permeability when baseline samples were compared with those obtained at Day 0 (three studies) [11,13,15]. and Day +7 (ten studies) [1,3,10–13,15,19,22,24]. Most studies also demonstrated a persistent increase in intestinal permeability at approximately Day +14 (seven studies) [11,13,15,18,19,22,24] and Day +30 (two studies) [3,18]. Although permeability began to return toward baseline between Day +21 and Day +30, it generally remained above pre-transplant levels [12].

Biomarker-based methodologies showed heterogeneity in intestinal permeability dynamics, in some cases even yielding conflicting findings. For several indirect biomarkers, such as REG3α, citrulline, ST2, IAP, LBP, I-BABP, and I-FABP, most studies showed trends similar to those observed with probe-based methodologies, with increased permeability between Day +7 and Day +14 and values returning toward baseline around Day +30 [2,3,7,8,10–13,15,20]. In contrast, for other biomarkers, such as occludin and calprotectin, no significant dynamic changes were identified [1,3]. Nevertheless, conflicting patterns were observed for some markers. Zonulin levels were elevated at baseline compared with reference values from a control group; however, relative to baseline, they decreased significantly at Day +7 (76.45 vs. 39.95; $p < 0.001$), Day +14 (76.45 vs. 43.00; p = NR), and Day +21 (76.45 vs. 41.45; $p < 0.001$), potentially suggesting improved barrier function [1]. Similarly, β2-defensin levels also decreased over the course of allo-HSCT compared with baseline at Day +7 (17.90 vs. 7.80; p = 0.101), Day +14 (17.90 vs. 4.31; p = NR), and Day +21 (17.90 vs. 6.20; p = 0.006) [1]. These discrepancies highlight the importance of assessing multiple biomarkers and permeability pathways simultaneously in order to obtain a comprehensive perspective [33]. Therefore, future studies should validate and identify the optimal methodologies for evaluating intestinal permeability in the allo-HSCT setting. Such studies should also delineate the main factors driving increased intestinal permeability, as a better understanding of these mechanisms would enable more accurate monitoring of its dynamics throughout the allo-HSCT course.

Factors associated with increased permeability during the allogeneic hematopoietic stem cell transplantation course

Throughout the allo-HSCT course, several factors may act individually or synergistically to modulate intestinal permeability. Key factors consistently evaluated in allo-HSCT studies include: 1) demographic characteristics (age, sex, underlying diagnosis) [1,3,7]; 2) transplant-related variables (conditioning regimen, use of total body irradiation, time since last chemotherapy) [1,3,7,9,20,21,23,24]; 3) dietary interventions [7,10,13,19,20]; 4) intestinal microbiota [3,20]; and 5) immune response (Table 2) [9,12,13]. Significant associations were found for the following factors: 1) conditioning regimen (four studies) [9,21,23,24], 2) Hematopoietic Cell Transplantation Comorbidity Index score (HCT-CI score: one study) [1], 3) dietary interventions (three studies) [7,10,20], 4) intestinal microbiota (two studies) [3,20] and 5) immune response (three studies) [9,12,13].

It is important to note that the conflicting or nonsignificant findings related to some of these factors may reflect limitations of the individual studies (such as lack of statistical power due to small sample sizes) as well as limitations of the methodologies themselves. Each of these factors has distinct underlying mechanisms that may affect different components of the gastrointestinal barrier, ultimately resulting in increased intestinal permeability. For instance, conditioning regimens can cause extensive enterocyte injury and increase both transcellular and paracellular permeability [8]. Because both pathways may be simultaneously affected, probe-based methodologies expressed as ratios may fail to detect such changes [8,28]. Indeed, among the six studies that evaluated the impact of conditioning regimens on intestinal permeability, the two that used probe-based ratios did not identify significant associations [1,3]. In contrast, the remaining four studies, which assessed permeability using 51Cr-EDTA, citrulline, and I-FABP, found that myeloablative conditioning was associated with increased intestinal permeability [9,21,23,24]. Taken together, these data emphasize the complexity of assessing intestinal permeability in the allo-HSCT setting. Additionally, as allo-HSCT increasingly shifts toward non-myeloablative conditioning regimens, there is a clear need to better characterize how intestinal permeability varies according to different chemotherapy protocols. Therefore, future studies should ideally use multiple complementary methodologies to capture the complex and dynamic changes that occur in the gastrointestinal barrier throughout the course of allo-HSCT.

An interesting finding of this review is the consistent association across studies between intestinal microbiota and intestinal permeability [3,20]. In the allo-HSCT setting, the intestinal microbiota has emerged as a key prognostic factor

Table 1 – Dynamics of intestinal permeability after allogeneic hematopoietic stem cell transplantation.

Intestinal Permeability Assay		Author, year n	Intestinal Permeability
Probe-based Methodology	L:R Ratio [3,11,13,15]	Wang 2023 [3] 80	↑ from preconditioning to Day +7, remained elevated at Day +30, and ↓ significantly between Day +7 and Day +30.
		Lutgens 2005 [15] 10	↑ from pre-transplant to post-transplant samples and ↓ between Day +14 and Day +21.
		Blijlevens 2004 [11] 32	↑ from pre-transplant to post-transplant samples and ↓ between Day +14 and Day +21.
		Blijlevens 2005 [13] 32	↑ from pre-transplant to post-transplant samples and ↓ between Day +14 and Day +21.
	L:M Ratio [1,10,12,18,19]	Tyszka 2022 [1] 45	↑ from pre-transplant to Day +7 samples.
		Blijlevens 2005 [10] 32	↑ from preconditioning to Day +7 samples and ↓ between Day +7 and Day +21.
		Borges 2020 [18] 31	↑ from preconditioning to Day +14 and Day +30 samples.
		Da Gama Torres 2008 [19] 53	↑ from admission to Day +6 and Day +14 samples.
		Pontoppidan 2015 [12] 30	↑ from pre-transplant to Day +7 samples. Similar values from pre-transplant to Day 0, Day +14, Day +21 and Day +28 samples.
	X:O Ratio [11,15]	Lutgens 2005 [15] 10	↑ from pre-transplant to Day 0, Day +7, Day +14 and Day +21 samples.
		Blijlevens 2004 [11] 32	↑ from pre-transplant to Day 0, Day +7, Day +14 and Day +21 samples. ↓ from Day +7 to Day +14 and Day +21 samples.
	R:O Ratio [11,15]	Lutgens 2005 [15] 10	↑ from pre-transplant to Day 0, Day +7, Day +14 and Day +21 samples.
		Blijlevens 2004 [11] 32	↑ from pre-transplant to Day +7, Day +14 and Day +21 samples.
	51Cr-EDTA [22–24]	Johansson 1997 [22] 25	↑ from pre-transplant to Day +1, Day +4, Day +7 and Day +10 samples.
		Johansson 2007 [23] 38	In patients with GVHD, intestinal permeability ↑ from baseline to Day –1. Subsequently, patients with grade 2–4 GVHD exhibited a further ↑ from Day –1 to Day +4, whereas permeability remained relatively stable in patients with grade 0–1 GVHD. In both groups, intestinal permeability remained stable at Day +7 and declined by Day +14, approaching baseline levels.
		Johansson 2001 [24] 17	Reduced-intensity conditioning No statistically significant changes in intestinal permeability were observed at any time point relative to baseline in patients receiving RIC. Myeloablative conditioning ↑ from pre-transplant to Day -1, Day +4, Day +7 and Day +14 samples.
Biomarker-based Methodology	REG3α [3,7]	Wang 2023 [3] 80	↑ from preconditioning to. Day +7 samples and ↓ from Day +7 to Day +30.
		Rashidi 2018 [7] 95	↑ at preconditioning samples when compared to healthy controls. ↓ from preconditioning to Day +7, followed by recovery through Day +28.
	ST2 [3]	Wang 2023 [3] 80	↑ from preconditioning to Day +7 and remained stable until Day +30.
	LBP [2,3,20]	Wang 2023 [3] 80	↑ from preconditioning to Day +7. ↓ from Day +7 to Day +30 ↑ from baseline to Day +21 samples in both the intervention and control groups.

Table 1 (*continued*)

Intestinal Permeability Assay	Author, year n	Intestinal Permeability
	Skaarud 2021 [20] 117†	
	Blijlevens 2005 [13] 32	↑ from preconditioning to Day 0, Day +7, day +14 and Day +21 samples. Peak at day +7.
	Ye 2012 [2] 73	LBP levels ↑ throughout the allo-HSCT course and remained above baseline through Day +100. In patients with GVHD, levels peaked at Day +14, whereas in patients without GVHD, the peak occurred at Day –1.
Occludin [3]	Wang 2023 [3] 80	Occludin levels remained similar across preconditioning, Day +7, and Day +30.
Claudin1 [3]	Wang 2023 [3] 80	↑ from preconditioning to Day +7, then remained stable at Day +30.
IAP [3]	Wang 2023 [3] 80	↓ from preconditioning to Day +7, with a further ↓ to Day +30.
I-BABP [8]	Derikx 2009 [8] 29	I-BABP levels decreased significantly over the study period, reaching a nadir at Day +0; however, by Day +21, levels had returned to values comparable to baseline.
I-FABP [7,8,20]	Skaarud 2021 [20] 117†	↓ from preconditioning to Day +21 samples in both the intervention and control groups.
	Rashidi 2018 [7] 95	↑ at preconditioning samples compared with healthy controls. Patients exhibited an initial ↓ from preconditioning to Day +7, followed by recovery through Day +28.
	Derikx 2009 [8] 29	I-FABP levels decreased significantly over the study period, reaching a nadir at Day +7; however, by Day +21, levels had returned to values comparable to baseline.
sCD14 [20]	Skaarud 2021 [20] 117†	Levels remained similar from preconditioning to Day +21.
Fecal Zonulin [1]	Tyszka 2022 [1] 32	↓ from preconditioning to Day +7, Day +14, and Day +21. ↑ at all allo-HSCT time points vs. reference values.
Beta-defensin-2 [1]	Tyszka 2022 [1] 14	Similar values from preconditioning to Day +7; ↓ at Day +14 and Day +21. ↓ at all allo-HSCT time points vs. reference values.
Calprotectin [1]	Tyszka 2022 [1] 31	Similar values from preconditioning to Day +7, Day +14 and Day +21. ↓ at all allo-HSCT time points vs. reference values.
Citrulline [7–13,15]	Pontoppidan 2015 [12] 30	↓ from preconditioning to Day 0, Day +7, Day +14, day +21, and Day +28.
	Blijlevens 2005 [10] 32	↓ from preconditioning to Day +7 and remained below baseline through Day +21.
	Blijlevens 2004 [11] 32	↓ from preconditioning to Day -6, Day 0, Day +7, Day +14, and Day +21.
	Blijlevens 2005 [13] 32	↓ from preconditioning to Day 0, Day +7, Day +14, and Day +21.
	Lutgens 2005 [15] 10	Overall, citrulline ↓ significantly over time, reaching a nadir at Day +5. Values comparable to baseline by Day +21.
	Rashidi 2018 [7] 95	↓ at preconditioning in allo-HSCT patients vs. healthy controls. ↓ from preconditioning to Day +7, followed by recovery through Day +28.
	Van der Velden 2010 [9] 86	↓ over the allo-HSCT course, remaining below baseline at Day +20. Only patients receiving cyclophosphamide and fludarabine conditioning achieved citrulline levels comparable to baseline by Day +20.
	Derikx 2009 [8] 29	↓ over allo-HSCT, nadir at Day +7, partial recovery by Day +21.
ZO-1 [32]	Nalle 2019 [32] 9	Patients with GVHD exhibited similar distribution of ZO-1 when compared to healthy controls.

Table 1 (continued)

Intestinal Permeability Assay	Author, year n	Intestinal Permeability
Actin [32]	Nalle 2019 [32] 9	Patients with GVHD exhibited similar distribution of actin when compared to healthy controls.
MLCK [32]	Nalle 2019 [32] 9	Patients with GVHD exhibited increased MLCK expression when compared to healthy controls.

51Cr-EDTA Absorption: Chromium-51–labeled ethylenediaminetetraacetic acid absorption; Allo-HSCT: Allogeneic hematopoietic stem cell transplantation; I-FABP: Intestinal fatty acid binding protein; LBP: LPS binding protein; ST2: Suppression of tumorigenicity-2 protein; sCD14: soluble cluster of differentiation; MLCK: long myosin light chain kinase; REG3: Regenerating family member-3 alpha; Not Reported; ZO: Zonula Occludens; †: this study investigated gut barrier function in response to a dietary intervention.

and therapeutic target, with mechanistic studies suggesting that altered intestinal permeability is one of the pathways through which the microbiota influences allo-HSCT outcomes [4,34]. This review identified that both intestinal microbiota diversity indices and microbiota composition are significantly associated with intestinal permeability [3,20]. In an allo-HSCT study including 80 patients, intestinal permeability was assessed using the L:R ratio [3]. An increased L:R ratio was significantly associated with a lower Shannon diversity index at Day +7 ($p = 0.044$) and Day +30 ($p = 0.047$). In addition, increased intestinal permeability was associated with a reduction in anaerobic organisms ($p = 0.020$) and with the presence of several specific bacterial species. Similarly, in another study including 117 patients, the Shannon diversity index was significantly associated with several intestinal permeability biomarkers: 1) I-FABP (baseline: $r = 0.31$; $p = 0.043$; Day +21: $r = 0.45$; $p = 0.005$), 2) LBP (Day +21: $r = -0.35$; $p = 0.032$), and 3) sCD14 (Day +21: $r = -0.35$; $p = 0.034$) [20]. Furthermore, the abundance of *Blautia* was significantly associated with I-FABP levels on Day +21 ($r = 0.39$; $p = 0.017$). These data highlight the close interplay between the intestinal microbiota and the gastrointestinal barrier and suggest that future therapeutic strategies targeting intestinal permeability may include modulation of the gut microbiota. Identifying potential therapeutic targets to modulate intestinal permeability is therefore of particular relevance, especially in light of its prognostic significance.

Prognostic significance of intestinal permeability

The prognostic significance of intestinal permeability in the allo-HSCT setting was evaluated in 14 studies including a total of 868 patients (Table 3) [1–3,7,11,13,15,18,20–23,35,36]. The most frequently assessed allo-HSCT–related outcomes were: 1) GVHD (ten studies with 769 patients) [1–3,7,18,20,21,23,35,36]; 2) infections (five studies with 387 patients) [1–3,13,36]; 3) overall survival (four studies with 385 patients) [3,18,20,36]; 4) non-relapse mortality (three studies with 369 patients) [7,20,36]; 5) oral mucositis (three studies with 109 patients) [1,11,13]; and 6) gastrointestinal toxicity (two studies with 35 patients) [15,22]. Additional outcomes included pulmonary complications (one study with 157 patients) [36] and transplant-associated thrombotic microangiopathy (TA-TMA; one study with 80 patients) [3].

Although GVHD was the most commonly evaluated outcome, its association with intestinal permeability remains inconsistent. Several studies using diverse permeability assessment methods (e.g., L:R ratio, L:M ratio, zonulin, I-FABP, LBP, sCD14, calprotectin, and Ussing chamber assays) and examining different clinical GVHD endpoints (incidence, gastrointestinal involvement, and severity) reported non-significant findings. Nevertheless, five (50%) of the ten available studies identified potentially relevant associations. For example, in a cohort of 45 patients, intestinal permeability was assessed at Day −7, Day +7, Day +14, and Day +21 using the L:M ratio, fecal zonulin, calprotectin, and β-defensin 2 [1]. In that study, no significant associations were observed between most markers and GVHD across the evaluated time points. However, calprotectin levels at Day +7 were significantly higher in patients who subsequently developed GVHD

Table 2 – Factors associated with intestinal permeability.

Type	Factor	Author, year n	Findings
Demographics [1,3,7]	Age [1,3,7]	Wang 2023 [3] 80	Baseline L:R ratios were similar regardless of age.
		Tyszka 2022 [1] 45	L:M ratios were similar regardless of age category.
		Rashidi 2018 [7] 95	Age was not associated with baseline levels of citrulline. Age was not associated with baseline levels of I-FABP. Age was not associated with baseline levels of Reg3α.
	Sex [1,3,7]	Wang 2023 [3] 80	Baseline L:R ratios were similar regardless of sex.
		Tyszka 2022 [1] 45	L:M ratios were similar regardless of sex.
		Rashidi 2018 [7] 95	Sex was not associated with baseline levels of citrulline. Sex was not associated with baseline levels of I-FABP. Sex was not associated with baseline levels of Reg3α.
	Underlying Diagnosis [3,7]	Wang 2023 [3] 80	Baseline L:R ratios were similar regardless of underlying diagnosis.
		Rashidi 2018 [7] 95	Citrulline levels were not associated with the underlying diagnosis. I-FABP levels were not associated with the underlying diagnosis. Reg3a levels were not associated with the underlying diagnosis.
Transplant-Related [1,3,7,9,20,21,23,24]	Conditioning Regimen [1,3,9,21,23,24]	Wang 2023 [3] 80	Conditioning regimen did not affect L:R trajectory.
		Tyszka 2022 [1] 45	L:M ratios were similar regardless of conditioning regimen.
		Johansson 2007 [23] 38	51Cr-EDTA was significantly higher in patients receiving myeloablative conditioning than in those receiving RIC on Day +4 and Day +7.
		Van der Velden 2010 [9] 86	Patients receiving myeloablative conditioning showed a rapid decline in citrulline levels within 10 days after initiation of chemotherapy, whereas those receiving non-myeloablative conditioning exhibited an early and short decrease in citrulline levels.
		Johansson 2001 [24] 17	While 51Cr-EDTA did not increase at any time point in patients receiving RIC, a statistically significant increase was consistently observed across all evaluated time points in patients receiving myeloablative conditioning.
		Lounder 2017 [21] 114	I-FABP levels were lower in patients receiving myeloablative regimen.
	Time Since Last Chemotherapy [7]	Rashidi 2018 [7] 95	Time since the last chemotherapy was not correlated with baseline intestinal citrulline levels. Time since the last chemotherapy was not correlated with baseline intestinal I-FABP levels. Time since the last chemotherapy was not correlated with baseline intestinal Reg3α levels.
	Intensity of Last Anti-Neoplastic Treatment Before Conditioning [7]	Rashidi 2018 [7] 95	Baseline citrulline levels were not associated with the intensity of the last anti-neoplastic treatment before conditioning. Baseline I-FABP levels were not associated with the intensity of the last anti-neoplastic treatment before conditioning. Baseline Reg3a levels were not associated with the intensity of the last anti-neoplastic treatment before conditioning.
	Total Body Irradiation [3]	Wang 2023 [3] 80	Total body irradiation did not affect L:R trajectory.
	HCT-CI [1,7,20]	Skaarud 2021 [20] 117†	There was no significant association between HCT-CI and baseline I-FABP and Day +21 I-FABP levels. There was no significant association between HCT-CI and baseline I-LBP and Day +21 I-LBP levels. There was no significant association between HCT-CI and baseline sCD14 and Day +21 sCD14 levels.

Table 2 (*continued*)

Type	Factor	Author, year n	Findings
		Tyszka 2022 [1] 45	Patients with higher HCT-CI scores demonstrated significantly higher baseline L:M ratios compared with those with lower scores.
		Rashidi 2018 [7] 95	Baseline citrulline levels were not associated with the HCT-CI score. Baseline I-FABP levels were not associated with the HCT-CI score. Baseline Reg3a levels were not associated with the HCT-CI score.
Dietary [7,10,13,19,20]	Dietary Intervention [7,10,13,19,20]	Skaarud 2021 [20] 117†	Baseline and Day +21 I-FABP levels were not statistically different between patients undergoing a dietary intervention and the control group. Baseline and Day +21 LBP levels were not statistically different between patients undergoing a dietary intervention and the control group. Day +21 sCD14 levels were higher in patients undergoing a dietary intervention compared with the control group.
		Da Gama Torres 2008 [19] 53	There was no significant difference in intestinal permeability between groups (glutamine vs. control groups).
		Blijlevens 2005 [10] 32	Citrulline levels did not differ significantly between the glutamine and placebo groups at preconditioning and Day +7. However, at Day +21, levels were higher in patients receiving glutamine. L:R ratio did not differ significantly between the glutamine and placebo groups.
		Blijlevens 2005 [13] 32	LBP levels did not differ significantly between the glutamine and placebo groups.
		Rashidi 2018 [7] 95	At Day +7, patients receiving TPN between Day 0 and Day +7 had significantly lower citrulline levels compared with those receiving enteral nutrition.
Intestinal Microbiota [3,20]	Intestinal Microbiome Diversity [3,20]	Wang 2023 [3] 80	⊠ L:R Ratio in patients with lower Shannon diversity index at Day +7 and Day +30.
		Skaarud 2021 [20] 117†	I-FABP levels were positively correlated with the Shannon index at Day +21 ** LBP levels were negatively correlated with the Shannon index at Day +21 ** sCD14 levels were negatively correlated with the Shannon index at Day +21 **
	Microbiota Composition [3,20]	Wang 2023 [3] 80	L:R ratios were significantly associated with the abundance of several bacterial species over the allo-HSCT course.
		Skaarud 2021 [20] 117†	I-FABP levels were positively correlated with *Blautia* abundance at Day +21 ** LBP levels were not significantly correlated with *Blautia* abundance ** sCD14 levels were not significantly correlated with *Blautia* abundance.
Immune System [9,12,13]	Inflammatory Markers [9,12,13]	Pontoppidan 2015 [12] 30	Citrulline was negatively associated with IL-6, but not with IL-10, IL-17A, TNF-⊠, CRP, microRNA-155, or microRNA-146a.
		Van der Velden 2010 [9] 86	Citrulline levels were significantly associated with CRP in multivariable analyses.
		Blijlevens 2005 [13] 32	CRP levels correlated significantly with LBP, the L:R ratio, and citrulline.

51Cr-EDTA Absorption: Chromium-51–labeled ethylenediaminetetraacetic acid absorption; CRP: C Reactive Protein; HCTI-CI: Hematopoietic Cell Transplantation Comorbidity Index; HSCT: Hematopoietic stem cell transplantation; I-FABP: Intestinal fatty acid binding protein; IL: Interleukin; L:M Ratio: Lactulose:Mannitol Ratio; L:R Ratio: Lactulose:Rhamnose Ratio; LBP: LPS binding protein; mL: milliliters; N: Number of patients included in this study; ng: nanograms; NR: Not Reported; NS: Not significant; OTU: Operational Taxonomic Unit; REG3: Regenerating family member-3 alpha; RIC: Reduced intensity conditioning; sCD14: soluble cluster of differentiation; TNF: Tumor necrosis factor; TPN: Total parenteral nutrition; yo: years old; **: only 47 patients provided stool samples for this analysis; †: this study investigated gut barrier function in response to a dietary intervention; ⊠: decreased; ⊠: increased.

Table 3 – Associations between intestinal permeability and allogeneic hematopoietic stem cell transplantation outcomes.

Outcome	Author, year n	Findings
GvHD [1–3,7,18,20,21,23,35,36]	Wang 2023 [3] 80	Baseline, Day +7, and Day +30 L:R ratios and zonulin genotypes were not associated with GVHD or gut-GvHD.
	Skaarud 2021 [20] 117†	Baseline and Day +21 I-FABP, LBP and sCD14 were not associated with GVHD.
	Borges 2020 [18] 31	There was no significant association between L:M Ratio and aGVHD.
	Rashidi 2020 [36] 157	There was no significant association between zonulin genotype and the cumulative incidence of aGVHD.
	Tyszka 2022 [1] 45	L:R ratio, zonulin, calprotectin, and beta-defensin-2 measures over the allo-HSCT course were not associated with GVHD, with the exception of calprotectin levels at Day +7.
	Johansson 2007 [23] 38	Patients with mild aGVHD (grade 0–1) exhibited significantly less disruption of intestinal permeability (measured by 51Cr-EDTA) on Day +4 when compared with patients with aGVHD grade 2–4.
	Rashidi 2018 [7] 95	Reg3α, I-FABP and citrulline measures over the allo-HSCT course were not associated with GVHD severity, with the exception of preconditioning citrulline levels.
	Ye 2012 [2] 73	Patients with aGVHD exhibited significantly higher LBP levels at Dy –1, Day +14, Day +21, and Day +28. Additionally, LBP levels were significant associated with the cumulative incidence of aGVHD at Day +7, Day +14, Day +21, and Day +27. An LBP level >15,000 was significantly associated with an increased risk of aGVHD in a multivariable Cox regression model.
	Coron 2021 [35] 19	Ussing chambers assay did not differ significantly between patients with aGVHD and those without aGVHD.
	Lounder 2017 [21 114	I-FABP levels below the median on Day 7 were associated with a higher incidence of GI GVHD.
TA-TMA [3]	Wang 2023 [3] 80	There was no significant association between TA-TMA and the L:R ratio at baseline, Day +7, or Day +30.
Infections [1–3,13,36]	Wang 2023 [3] 80	There was no significant association between BSI and the L:R ratio at baseline, Day +7, or Day +30. Zonulin genotypes were also not associated with BSI.
	Tyszka 2022 [1] 45	There was no significant association between infections and several intestinal permeability markers (L:M ratio, zonulin, calprotectin, and beta-defensin-2) measured at Day –7, Day +7, Day +14, and Day +21, or with the ΔL:M ratio.
	Rashidi 2020 [36] 157	There was no significant association between haptoglobin genotype and the 100-day cumulative incidence of BSI or CDI.
	Blijlevens 2005 [13] 32	There was no significant association between bacteremia and LBP levels at Day -12, and Day 0.
	Ye 2012 [2] 73	In patients with no aGVHD or grade I aGVHD, infections were significantly associated with LBP levels over the allo-HSCT course.
Overall Survival [3,18,20,36]	Wang 2023 [3] 80	Zonulin genotypes were not associated with mortality *
	Borges 2020 [18] 31	There was no significant association between intestinal permeability and mortality.
	Rashidi 2020 [36] 157	There was no significant association between overall survival and haptoglobin genotype.
	Skaarud 2021 [20] 117†	There was no significant association between mortality and several intestinal permeability markers (I-FABP, LBP, and sCD14) measured at baseline and Day +21.
NRM [7,20,36]	Skaarud 2021 [20] 117†	There was no significant association between NRM and several intestinal permeability markers (I-FABP, LBP, and sCD14) measured at baseline and Day +21.
	Rashidi 2020 [36] 157	There was no significant association between NRM and haptoglobin genotype.

Table 3 (continued)

Outcome	Author, year n	Findings
	Rashidi 2018 [7] 95	There was a significant association between NRM and Reg3α levels measured at preconditioning, Day +7, and Day +28. No significant association was observed between NRM and I-FABP or citrulline levels.
Oral Mucositis [1,11,13]	Tyszka 2022 [1] 45	There was no significant association between oral mucositis and several intestinal permeability markers (L:M ratio, zonulin, calprotectin, and β-defensin-2) measured at Day −7, Day +7, Day +14, and Day +21. The only exception was zonulin levels at Day +14, which were associated with oral mucositis.
	Blijlevens 2004 [11] 32	There was no significant association between mucositis and any of the probe-based assays (L:R, O:R, and X:R ratios).
	Blijlevens 2005 [13] 32	The peak of oral mucositis coincided with the nadir of citrulline concentrations.
Gastrointestinal toxicity [15,22]	Johansson 1997 [22] 25	There was a significant association between the severity of gastrointestinal toxicity and intestinal permeability. Also, cumulative gastrointestinal toxicity was positively correlated with mean relative intestinal permeability over the 14-day post-HSCT period.
	Lutgens 2005 [15] 10	There was a significant association between gastrointestinal toxicity and citrulline nadir. However, gastrointestinal toxicity was not associated with probe-based methods (L:R, X:O, or R:O ratios).
Pulmonary Complications [36]	Rashidi 2020 [36] 157	There was no significant association between haptoglobin genotype and the 100-day cumulative incidence of pulmonary complications, and cumulative incidence of idiopathic pulmonary syndrome.

Allo-HSCT: Allogeneic hematopoietic stem cell transplantation; BSI: Bloodstream infection; HP: Haptoglobin; I-FABP: Intestinal fatty acid binding protein; LBP: LPS binding protein; sCD14: soluble cluster of differentiation; GVHD: Graft versus host disease; TA-TMA: Transplant associated thrombotic microangiopathy; N: Number of patients; NRM: Non-relapse mortality; *: The zonulin genotype analysis included a different cohort of 116 patients undergoing allo-HSCT; †: this study investigated gut barrier function in response to a dietary intervention.

compared with those who did not (64.758 vs. 14.060; p = 0.040). In another study including 38 patients, those who developed severe GVHD (grade II–IV) exhibited significantly higher intestinal permeability (measured by 51Cr-EDTA) at Day +4 when compared with patients who did not develop this complication (7 vs. 11; p = 0.02) [23]. Preconditioning citrulline levels were also significantly associated with severe GVHD (grade II–IV: p < 0.01; grade III–IV: p = 0.03) in a study including 95 patients [7]. Despite these conflicting findings, the available evidence suggests that intestinal permeability may play a prognostic role in GVHD [1,2,7,21,23]. However, further clarification will require future studies with larger sample sizes and the concomitant assessment of multiple intestinal permeability markers.

Infections represent another clinically relevant endpoint, particularly in light of studies on the intestinal microbiota suggesting that bacterial translocation across the gut barrier may contribute to the development of bloodstream infections (BSI) [4,6,37]. In the allo-HSCT setting, however, among the five studies evaluating this outcome, only one reported a significant association. In that study, which included 73 patients, LBP levels were significantly higher in patients with no or mild GVHD (grade I) who developed infections compared with those who did not (p < 0.001) [2]. In contrast, within the same cohort, LBP levels were not significantly associated with infections among patients with grade II GVHD [2]. Similarly, in the remaining four studies, infections were not significantly associated with other intestinal permeability biomarkers, including the L:R ratio, fecal zonulin, L:M ratio, calprotectin, β-defensin 2, and haptoglobin genotype [1,3,13,36]. For the other clinical outcomes, conflicting and/or predominantly non-significant findings were also reported. With respect to overall survival, all available studies consistently showed no significant association with intestinal permeability [3,18,20,36]. For non-relapse mortality, among the three included studies, only one identified a significant association between REG3α levels and this outcome at the preconditioning time point (9.91 vs. 6.23; p = 0.02), Day +7 (11.51 vs. 4.20; p < 0.001), and Day +28 (17.33 vs. 10.47; p = 0.03) [7]. Predominantly non-significant associations were also reported for oral mucositis, with only one study demonstrating a significant relationship between fecal zonulin levels and this outcome at Day +14 (41.125 vs. 43.000; p = 0.048)[1]. For pulmonary complications and TA-TMA, no significant associations with intestinal permeability were identified [3,36].

Overall, these findings indicate that although intestinal permeability is frequently altered in patients undergoing allo-HSCT, its prognostic significance remains inconsistent across studies. While several investigations have reported associations between intestinal permeability and allo-HSCT–related outcomes, the available evidence is inconclusive or of limited methodological quality or both. It is possible that, for each specific clinical endpoint, a particular intestinal permeability biomarker may offer greater prognostic accuracy.

Future directions and conclusions

Although significant advances have been made in understanding the role of intestinal permeability in the allo-HSCT setting, much remains to be elucidated. Clear conclusions

regarding the dynamics and prognostic significance of intestinal permeability during allo-HSCT remain elusive due to differences in study design, variability in biomarker assays and sampling time points, lack of adjustment for potential confounders (such as diet, medication use, and comorbidities), and the absence of comparisons with a standardized gold-standard test (Ussing chambers). Notwithstanding these limitations, this comprehensive narrative review highlights the potential role of intestinal permeability as a prognostic variable throughout the allo-HSCT course that can be monitored to guide risk stratification strategies and future therapeutic interventions. To further advance this field, future studies should perform longitudinal assessments using multiple intestinal permeability methodologies to identify the most relevant pathways and modulators of gut barrier function during allo-HSCT. Simultaneous measurement of several permeability biomarkers may better capture the heterogeneity of allo-HSCT patients. In addition, future studies should aim to systematically identify and account for all potential confounders that modulate intestinal permeability in the allo-HSCT setting, both to improve analytical accuracy and to explore them as potential therapeutic targets. Finally, mechanistic studies are needed to further elucidate the pathways through which increased intestinal permeability may influence allo-HSCT-related outcomes, including immune modulation and bacterial translocation.

Authors contributions

All authors contributed significantly to the development of this study and manuscript. G.L.V.d.O., A.S.F.J., and B.F.R.S. conceived and designed the study with input from other authors. A.S.F.J. performed the literature search, the title and abstract screening and full-text review. A.S.F.J. and B.F.R.S. performed the data abstraction and wrote the first manuscript draft. All authors critically reviewed, edited and collectively approved the final manuscript file.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest

The authors declare no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.htct.2026.106541.

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Supplementary Files

Supplemental Search Strategy

(((((("Intestinal Barrier Function") OR ("Intestinal Permeability")) OR ("Zonuline")) OR ("Gut Barrier")) AND ("Stem Cell Transplantation"[Mesh] OR "Bone Marrow Transplantation"[Mesh] OR (("stem cell"[tiab] OR "stem cells"[tiab] OR "bone marrow"[tiab]) AND (transplant[tiab] OR transplants[tiab] OR transplanted[tiab] OR transplanting[tiab] OR transplantation[tiab] OR transplantations[tiab] OR post-transplant[tiab] OR post-transplants[tiab] OR post-transplantation[tiab] OR post-transplantations[tiab] OR post-transplanted[tiab] OR posttransplant[tiab] OR posttransplants[tiab] OR posttransplanted[tiab] OR posttransplantation[tiab] OR posttransplantations[tiab])) OR ASCT[tiab] OR HSCT[tiab] OR Allo-HSCT[tiab] OR AlloHSCT[tiab]))

Supplementary Table 1: Articles identified through manual review of reference lists

Author, year	Decision
Rashidi 2018 [1]	Include
Ye 2012 [2]	
Van der Velden 2010 [3]	
Blijlevens 2005 [4]	
Lutgens 2005 [5]	
Blijlevens 2004 [6]	
Derikx 2009 [7]	
Blijlevens 2005 [8]	
Nalle 2014 [9]	Exclude Animal model study
Hill 1998 [10]	
Brown 1999 [11]	
Noth 2011 [12]	
Hanash 2012 [13]	
Lehnert 1986 [14]	
Lehnert 1994 [15]	
Sykes 198 [8 [16]	
Koltun 1996 [17]	
Takashima 2011 [18]	
Lutgens 2003 [19]	
Hill 1997 [20]	
Keefe 1997 [21]	Exclude Non allo-HSCT study*
Sundstrom 1998 [22]	
Elting 2003 [23]	
Keefe 2000 [24]	
Inutsuka 2003 [25]	
Fang Yan 2007 [26]	
Fang Yan 2011 [27]	
Furusawa 2013 [28]	

Scott 2020 [29]	
Sapone 2006 [30]	
Tripathi 2009 [31]	
Papp 2008 [32]	
Márquez 2012 [33]	
Hamarneh 2014 [34]	
Liu 2016 [35]	
Parlato 2018 [36]	
Zeissig 2007 [37]	
Weber 2008 [38]	
Bertiaux-Vandaële 2011 [39]	
Marchiando 2011 [40]	
Zhao 2016 [41]	
Lasek-Bal 2020 [42]	
Olsson 2021 [43]	
Ellis 2021 [44]	
Bjarnason 1983 [45]	
Selby 1987 [46]	
Jenkins 1992 [47]	
Clayburgh 2005 [48]	
Rosenblatt 2001 [49]	
Madara 1990 [50]	
Wang 2005 [51]	
Graham 2006 [52]	
Al-Sadi 2008 [53]	
Garcia 1995 [54]	
Garcia 1998 [55]	
Smecuol 2001 [56]	
Blijlevens 2005 [57]	
Meddings 1998 [58]	

Blijlevens 2004 [59]	
Pytlík 2002 [60]	
Lutgens 2004 [61]	
Crenn 2008 [62]	
Papadopoulou 1996 [63]	
Ferry 1989 [64]	
Taur 2012 [65]	Exclude Intestinal permeability not assessed
Montassier 2014 [66]	
Slavin 1998 [67]	
Khoury 2001 [68]	
Storb 1983 [69]	
Panoskaltsis-Mortari 1998 [70]	
Nestel 1992 [71]	
Haak 2018 [72]	
Rapoport 1999 [73]	
Sonis 2001 [74]	
Van Zaanen 1994 [75]	
Harris 2016 [76]	
Cheney 1987 [77]	
Ziegler 1992 [78]	
Schloerb 1999 [79]	
Tamburini 2018 [80]	
Mehta 2017 [81]	
Wardill 2012 [82]	Exclude Non-original Research
Valitutti 2019 [83]	
Lallès 2019 [84]	
Schulzke 2009 [85]	
De Mooij 2017 [86]	
Bjarnason 1995 [87]	
Bjarnason 1994 [88]	

Melichar 2011 [89]	
Shallis 2018 [90]	
Peled 2016 [91]	
Blijlevens 2000 [92]	
Hill 2000 [93]	
Ferrara 2009 [94]	
Tsukita 2001 [95]	
Blijlevens 2005 [96]	
De-Souza 2005 [97]	
Peterson 2004 [98]	
Couriel 2004 [99]	
Carpenter 2017 [100]	
Mahendra 1994 [101]	Full text not found

Allo-HSCT: Allogeneic hematopoietic stem cell transplantation; *: Studies that did not report data specifically for patients undergoing allo-HSCT were also excluded for this reason

Supplementary Table 2: General data of studies evaluating intestinal permeability in the allo-HSCT setting

Author, year **n (Population)**	**Gut Barrier Detection Methods**	**Assessment Timing**
Wang 2023 [102] 80 (Adults and pediatrics)	L:R Ratio, LBP, REG3α, ST2, Occludin, IAP, and Claudin1	Preconditioning, Day +7, and Day +30
Skaarud 2021 [103] 117 (Adults)	I-FABP, LBP, and sCD14	Baseline, and Day +21
Tyszka 2022 [104] 51 (Adults)	L:M Ratio, Zonulin, Calprotectin, and Beta-defensin-2	L:M Ratio: Day -7, and Day +7 Zonulin, calprotectin and Beta-defensin-2: Day -7, Day +7, Day +14, and Day +21
Pontoppidan 2015 [105] 30 (Adults and pediatrics)	L:M Ratio, and Citrulline	Baseline, Day 0, Day +7, Day +14, Day +21, and Day +28
Nalle 2019 [106] 9 (NR)	ZO-1, Actin, and MLCK	Two or more weeks after allo-HSCT (specific date NR)
Johansson 1997 [107] 25 (Adults)	51Cr-EDTA	Preconditioning, 2D after starting conditioning regimen, Day +1, Day +4, Day +7, Day +10, and Day +14

Johansson 2007 [108] 38 (Adults)	51Cr-EDTA	Preconditioning, Day -1, Day +4, Day +7, and Day +14
Johansson 2001 [109] 17 (Adults)	51Cr-EDTA	Preconditioning, Day -1, Day +4, Day +7, and Day +14
Rashidi 2018 [1] 95 (Adults)	Citrulline, I-FABP, and REG3α	Preconditioning, Day +7, and Day +28
Ye 2012 [2] 73 (Adults and pediatrics)	LBP	Day -9, Day 0-1, Day +7, Day +14, Day +21, Day +28, Day +56, Day +90-100, At the onset of aGvHD, and at the time of aGvHD remission.
Lounder 2017 [110] 114 (Adults and pediatrics)	I-FABP	Day +7
Derikx 2009 [7] 29 (Adults)	I-FABP, I-BABP, and Citrulline	Day -12, Day -6, Day 0, Day +7, Day +14, and Day +21
Van der Velden 2010 [3] 86 (Adults)	Citrulline	Three times weekly from hospital admission to Day +20.
Borges 2020 [111] 31 (Adults and pediatrics)	L:M Ratio	Preconditioning, Day +14, and Day +30
Coron 2021 [112] 19 (Adults)	Ussing chambers assay	Between Day +21 and Day +28
Da Gama Torres 2008 [113] 53 (Adults)	L:M Ratio	On admission, Day +6, and Day +14
Rashidi 2020 [114] 157 (Adults and pediatrics)	Haptoglobin genotype	Between admission and Day 0

Blijlevens 2005 [4] 32 (Adults)	Citrulline, and L:R Ratio	Day -12, Day -6, Day 0, Day +7, Day +14, and Day +21
Lutgens 2005 [5] 10 (Adults)	Citrulline*, X:O Ratio, R:O Ratio, and L:R Ratio	Day -12, Day -7, Day 0, Day +7, Day +14, and Day +21
Blijlevens 2004 [6] 32 (Adults)	Citrulline, L:R Ratio, R:O Ratio, and X:O Ratio	Preconditioning, Day -12, Day -6, Day 0, Day +7, Day +14, and Day +21
Blijlevens 2005 [8] 32 (Adults)	Citrulline, LBP, L:R Ratio, R:O Ratio, and X:O Ratio	Day -12, Day -6, Day 0, Day +7, Day +14, and Day +21

51Cr-EDTA Absorption: Chromium-51–labeled ethylenediaminetetraacetic acid absorption; aGvHD: Acute graft-versus-host disease; Allo-HSCT: Allogeneic hematopoietic stem cell transplantation; D: Day; I-BABP: Ileal bile acid–binding protein; I-FABP: Intestinal fatty acid–binding protein; IAP: Intestinal alkaline phosphatase; L: Lactulose; LBP: Lipopolysaccharide-binding protein; M: Mannitol; MLCK210: Myosin light chain kinase 210 isoform; N: Number of patients included in the study; NR: Not reported; O: 3-O-methyl-Day -glucose; R: Rhamnose; REG3α: Regenerating islet-derived protein 3 alpha; sCD14: Soluble cluster of differentiation 14; ST2: Suppression of tumorigenicity 2; X: Xylose; *: citrulline was assessed daily from Day -6 to Day +1, every other day until Day +7, daily until Day +12 and finally on Day +21

Supplementary Table 3: Current Methods and Biomarkers for the Assessment of Intestinal Barrier Dysfunction

Methodologies		Interpretation	Advantages	Disadvantages
Probe-based Methodologies	L:M Ratio [102,104,105, 111,115–117]	↑ L:M ratio → ↑ permeability ↓ L:M ratio → ↓ permeability	Direct intestinal permeability marker.	Requires the patient to drink an oral solution. Not appropriate for uncooperative patients (critically ill). Not well tolerated in patients with nausea or vomiting. Not suited to evaluate colonic permeability – lactulose and mannitol are degraded by the colonic microbiota.

				Controversy regarding the predominant route of mannitol absorption (paracellular vs. transcellular).
	L:R Ratio [102,115,117,118]	↑ L:R ratio → ↑ permeability ↓ L:R ratio → ↓ permeability	Direct intestinal permeability marker. ↓Risk of food-related contamination of results. ↓LR solution volume and dose. Well-tolerated and validated in pediatric patients. Good correlation	Not well tolerated in patients with nausea, mucositis or vomiting. Not suited to evaluate colonic permeability – lactulose is degraded by the colonic microbiota.

			with enteritis markers.	
	X:O Ratio [5,117,119]	↑ X:O Ratio → ↓ permeability ↓ X:O Ratio → ↑ permeability	Marker of intestinal absorptive capacity.	Does not evaluate the paracellular route.
	R:O Ratio [5,117,119]	↑ R:O Ratio → ↓ permeability ↓ R:O Ratio → ↑ permeability	Marker of intestinal absorptive capacity.	This assay does not evaluate the paracellular route.
	51-CrEDTA Absorption [6,104,108,114,115, 117,120–122]	↑ Urinary excretion of 51CrEDTA → ↑ Paracellular pathway impairment.	Marker of total gastrointestinal permeability. Easily detectable. Not degraded by colonic bacteria.	Impact on menstrual cycle. Radioactivity.
	Ussing chambers assay [112,117]	↓ TEER → ↑ Intestinal permeability (paracellular route).	Examination of specific intestinal segments separately. Gold standard technique.	May require specialized expertise and equipment. Time and labor-intensive.

			Allows measurement of both paracellular and transcellular route.	
Biomarker-based Methodology	Citrulline [62,104,105]	↓ Plasma levels → ↓ Functional enterocyte mass.	Simple and reliable surrogate of enterocyte mass.	Not valid in cases of renal failure.
	Zonulin [104,115,117,123–125]	↑ Zonulin → Impairs enterocyte tight junction function → ↑ Paracellular transport → ↑ Permeability.	It is one of the most reliable serum markers for "leaky gut syndrome".	It is not the sole mediator of paracellular pathway opening. Concerns regarding the validity and specificity of zonulin ELISA kits. Gluten, circadian rhythm and the intestinal microbiota can

				modulate the release of zonulin. Does not directly assess enterocyte damage. ↓ Zonulin levels do not necessarily indicate intact barrier integrity.
	I-FABP [7,115,125–128]	↑ I-FABP → ↑ Damage to enterocytes of the small and large intestine.	Promising in the early diagnosis of intestinal ischemia.	Can also originate from lesions in other organs. Does not allow accurate assessment of enterocyte loss in the jejunum and ileum.
	I-BABP [7]	↑ I-BABP → ↑ Damage to enterocytes of the jejunum and ileum.	Assessment of jejunum and ileum permeability.	Does not allow for accurate assessment of enterocyte loss in the small

				and large intestine.
	LBP [115,125,129,130]	↑ LBP → ↑ Intestinal permeability.	A reliable indirect marker of intestinal permeability. Previously explored in other clinical settings.	May be elevated in patients with chronic liver disease and other conditions.
	Calprotectin [104,129,131]	Indirect marker of intestinal permeability. ↑ Calprotectin → ↑ intestinal inflammation → ↑ intestinal permeability.	Sensitive to inflammation of the intestinal mucosa.	Primarily a marker of inflammation rather than intestinal barrier function.
	Beta-defensin-2 [104,132,133]	↓ Levels →↑ Damage to the intestinal epithelium and disruption of the intestinal barrier.	Marker of intestinal inflammation. Explored as a predictive marker of GvHD.	Does not directly reflect the integrity of the intestinal barrier.
	sCD14 [125,134,134,135]	↑ sCD14 → Systemic inflammatory response (immune activation).	Explored as a marker to aid in the early diagnosis and prognostic	Limitations regarding cutoff values. Levels may vary in the

			evaluation of sepsis.	elderly and patients with renal failure.
	MLCK [106]	Its upregulation leads to increased barrier permeability.	Associated with GVHD pathogenesis.	Requires invasive biopsies.
	REG3α [136,137]	↑ Plasma levels→ ↑ Intestinal injury.	Biomarker of GVHD in the lower GI tract.	Does not directly reflect intestinal permeability.
	ST2 [138,139]	↑ ST2 → ↑ immune response and ↑ intestinal permeability.	Explored as a predictor of treatment-resistant GvHD and non-relapse mortality.	Reliable cut-off points are still unclear.
	IAP [140]	↓ IAP → ↓ Intestinal junction proteins → ↑ Permeability.	Excellent marker for crypt-villus differentiation.	Influenced by enteral nutritional intake.
	Claudin-3 [141]	Form a dense structure between intestinal epithelial cells to reduce the exchange of permeable material.	A surrogate marker of intestinal permeability.	Indirect measure of intestinal permeability. Surrogate especially of paracellular route.
	Occludin [102,117]	↓ Occludin → ↑ Permeability.	Reflects structural	It may not directly reflect

		Decreased levels of occludin reflect disorganized tight junctions and loss of epithelial barrier integrity.	damage to the epithelial barrier.	functional changes in the barrier.
	Haptoglobin genotype [114,142]	Homozygous HP1-1: not able to produce zonulin. HP2-1 or HP2-2: able to produce zonulin.	HP2-2 previously associated with chronic GvHD.	Does not allow assessment of the longitudinal dynamics of intestinal permeability.

51Cr-EDTA Absorption: Chromium-51–labeled ethylenediaminetetraacetic acid absorption; ELISA: Enzyme-linked immunosorbent assay; GI: Gastrointestinal; HP: Haptoglobin; I-BABP: Ileal bile acid–binding protein; I-FABP: Intestinal fatty acid–binding protein; IAP: Intestinal alkaline phosphatase; L:M Ratio: Lactulose:Mannitol ratio; L:R Ratio: Lactulose:Rhamnose ratio; LBP: Lipopolysaccharide-binding protein; LPS: Lipopolysaccharide; MLCK: Myosin light chain kinase 210 isoform; O: 3-O-methyl-Day -glucose; REG3α: Regenerating islet-derived protein 3 alpha; sCD14: Soluble cluster of differentiation 14; ST2: Suppression of tumorigenicity 2; TEER: Transepithelial electrical resistance; X: Xylose; ↑ : Increase; ↓: Decrease

Supplementary Table 4: Articles highlighting correlations between intestinal permeability methodologies

Author, year **n**	**Methodologies**	**Findings**
Rashidi 2018 [1] 95	Citruline, I-FABP, and REG3a	There were no correlations between pre-HSCT biomarkers except for a weak correlation between I-FABP and REG3a (r = -0.23; 95% CI 0.03-0.42).
Derikx 2009 [7] 29	I-FABP, and I-BABP	I-FABP and I-BABP levels were significantly correlated (r = 0.436, p < 0.0001).
Lutgens 2005 [5] 10	Citrulline, L:R Ratio, X:O Ratio, and R:O Ratio	Exhibited distinct trajectories. Citrulline indicated maximal damage at Day +7, whereas probe-based methodologies indicated maximal damage at later time points (Day +14 to Day +21). Significant correlation only between citrulline and the R:O ratio (p = 0.025).
Blijlevens 2004 [6] 32	Citrulline, L:R Ratio, X:O Ratio, and R:O Ratio	Citrulline levels correlated significantly with the L:R ratio (r = −0.41), X:O ratio (r = 0.24), and R:O ratio (r = 0.41).

Wang 2023 [102] 80	L:R Ratio, LBP, REG3α, ST2, Occludin, IAP, and Claudin1	The L:R ratio correlated with LBP levels ($r^2 = 0.208$; $P = 0.0014$). The trajectories of plasma REG3α and ST2 were similar to that of the L:R ratio.
Tyszka 2022 [104] 51	L:M Ratio, Zonulin, Calprotectin, and Beta-defensin-2	No significant associations were observed between intestinal permeability methodologies.

HSCT: hematopoietic stem cell transplant; I-BABP: ileal bile acid–binding protein; I-FABP: intestinal fatty acid–binding protein; IAP: intestinal alkaline phosphatase; L:M Ratio: lactulose:mannitol ratio; L:R Ratio: lactulose:rhamnose ratio; LBP: lipopolysaccharide-binding protein; N: number of patients; O: 3-O-methyl-Day -glucose; REG3α: regenerating islet-derived protein 3 alpha; ST2: suppression of tumorigenicity; X: xylose

Supplementary Table 5: The Dynamics of Intestinal Permeability over the Allo-HSCT

Intestinal Permeability Assay		**Author, year n**	**Assessment Timing**	**Intestinal Permeability**
Probe-based Methodology	L:R Ratio [5,6,8,102]	Wang 2023 [102] 80	Pre-conditioning, Day +7, and Day +30	Preconditioning vs. Day +7 ↑ L:R ratio on Day +7 (0.19 vs. 0.86; $p < 0.0001$). Preconditioning vs. Day +30 ↑ L:R ratio on Day +30 (0.19 vs. 0.40; $p < 0.014$). Day +7 vs. Day +30 ↓ L:R ratio on Day +30 (0.86 vs. 0.40; $p = 0.014$).
		Lutgens 2005 [5] 10	Day -12 Day -7 Day 0 Day +7 Day +14 Day +21	Day -12: 0.04 (95% CI 0.03-0.06). Day -7: 0.05 (95% CI 0.03-0.07). Day 0: 0.13 (95% 0.00-0.27). Day +7: 0.12 (95% CI 0.06-0.17). Day +14: 0.18 (95% CI 0.02-0.34). Day +21: 0.13 (95% CI 0.06-0.20). All Samples Compared with baseline values, the ratio was significantly increased at Day +7 ($p = 0.020$) and Day +21 ($p = 0.012$).
		Blijlevens 2004 [6] 32	Pre-conditioning Day -12 Day -6	Day -12: 0.04 (95% CI 0.04-0.05). Day -6: 0.04 (95% CI 0.03-0.06). Day 0: 0.10 (95% CI 0.06-0.14). Day +7: 0.18 (95% CI 0.14-0.22).

			Day 0 Day +7 Day +14 Day +21	Day +14: 0.20 (95% CI 0.11-0.30). Day +21: 0.26 (95% CI 0.01-0.51). Preconditioning vs. Day 0 There was a significant increase in the L:R ratio at Day 0 ($p < 0.0001$).
		Blijlevens 2005 [8] 32	Day -12 Day -6 Day 0 Day +7 Day +14 Day +21	Day -12: 0.04 (95% CI 0.04-0.05). Day -6: 0.04 (95% CI 0.03-0.06). Day 0: 0.10 (95% CI 0.06-0.14). Day +7: 0.18 (95% CI 0.14-0.22). Day +14: 0.20 (95% CI 0.11-0.30). Day +21: 0.26 (95% CI 0.01-0.51). Preconditioning (Day -12) vs. Day 0 There was a significant increase in the L:R ratio at Day 0 ($p < 0.0001$). Preconditioning (Day -12) vs. Day +7 There was a significant increase in the L:R ratio at Day +7 ($p < 0.005$). Preconditioning (Day -12) vs. Day +14 There was a significant increase in the L:R ratio at Day +14 ($p < 0.005$). Preconditioning (Day -12) vs. Day +21 There was a significant increase in the L:R ratio at Day +21 ($p < 0.005$).

	L:M Ratio [4,104,105, 111,113]	Tyszka 2022 [104] 45	Day -7 Day +7	Day -7 vs. Reference values ↑ L:M ratio when compared to reference values (0.04 vs. 0.035) ↑ L:M ratio in 53% of patients even before the conditioning regimen (median value 0.190). Day +7 vs. Reference values ↑ L:M ratio in 76% of patients on Day +7 (median value 0.242). Day -7 vs. Day +7 ↑ L:M ratio on Day +7 (0.04 vs. 0.16; p <0.01)
		Blijlevens 2005 [4] 32	Pre-conditioning Day +7 Day +21	Preconditioning Glutamine group: 0.04 (±0.02). Placebo group: 0.04 (±0.02). Day +7 Glutamine group: 0.15 (±0.07). Placebo group: 0.19 (±0.08). Day +21 Glutamine group: 0.11 (±0.14). Placebo group: 0.17 (±0.13).
		Borges 2020 [111] 31	Pre-conditioning Day +14 Day +30	All Samples vs. Controls Preconditioning: 24% of patients exhibited an L:M ratio exceeding 2 SD above that of healthy control samples.

				Day +14: 60% of patients exhibited an L:M ratio exceeding 2 SD above that of healthy control samples. Day +30: 27% of patients exhibited an L:M ratio exceeding 2 SD above that of healthy control samples. All Samples Preconditioning: 0.0127. Day +14: 0.092. D:30: 0.141. Intestinal permeability increased, although the change did not reach statistical significance in a paired Student's t-test.
		Da Gama Torres 2008 [113] 53	On admission Day +6 Day +14	All Samples Admission: 0.812±0.650 Day +6: 1.460±0.655 Day +14: 1.974±0.878 There were significant rises in the ratio from admission to Day +14 ($p < 0.001$).
		Pontoppidan 2015 [105] 30	Baseline (7 to 14 days prior to allo-HSCT) Day 0 Day +7 Day +14 Day +21 Day +28	Baseline vs. Day 0 Similar L:M Ratios (values NR). Baseline vs. Day +7 ↑ L:M ratio on Day +7 (values NR, $p < 0.001$) Baseline vs. Day +14

				Similar L:M Ratios (values NR). Baseline vs. Day +21 Similar L:M Ratios (values NR). Baseline vs. Day +28 Similar L:M Ratios (values NR).
	X:O Ratio [5,6]	Lutgens 2005 [5] 10	Day -12 Day -7 Day 0 Day +7 Day +14 Day +21	Day -12: 0.68 (95% CI 0.56-0.80). Day -7: 0.65 (95% CI 0.52-0.79). Day 0: 0.61 (95% CI 0.45-0.77). Day +7: 0.51 (95% CI 0.47-0.57). Day +14: 0.51 (95% CI 0.43-0.58). Day +21: 0.45 (95% CI 0.33-0.58).
		Blijlevens 2004 [6] 32	Pre-conditioning Day -12 Day -6 Day 0 Day +7 Day +14 Day +21	Day -12: 0.64 (95% CI 0.59-0.70). Day -6: 0.59 (95% CI 0.54-0.65). Day 0: 0.57 (95% CI 0.50-0.63). Day +7: 0.40 (95% CI 0.31-0.49). Day +14: 0.44 (95% CI 0.38-0.49). Day +21: 0.45 (95% CI 0.34-0.57).
	R:O Ratio [5,6]	Lutgens 2005 [5] 10	Day -12 Day -7 Day 0 Day +7 Day +14 Day +21	Day -12: 0.23 (95% CI 0.17-0.26). Day -7: 0.29 (95% CI 0.22-0.35). Day 0: 0.21 (95% CI 0.15-0.28). Day +7: 0.13 (95% CI 0.10-0.16). Day +14: 0.13 (95% CI 0.07-0.20). Day +21: 0.09 (95% CI 0.07-0.20). All Samples Compared with baseline, the R/O ratio was significantly decreased at Day +7

				(p = 0.027), Day +14 (p = 0.033), and Day +21 (p = 0.036).
		Blijlevens 2004 [6] 32	Preconditioning Day -12 Day -6 Day 0 Day +7 Day +14 Day +21	Day -12: 0.25 (95% CI 0.22-0.27). Day -6: 0.24 (95% CI 0.21-0.27). Day 0: 0.25 (95% CI 0.15-0.36). Day +7: 0.11 (95% CI 0.05-0.18). Day +14: 0.11 (95% CI 0.07-0.14). Day +21: 0.11 (95% CI 0.07-0.15).
	51Cr-EDTA [107–109]	Johansson 1997 [107] 25	Prior to Conditioning 2d after conditioning regimen initiation Day +1 Day +4 Day +7 Day +10 Day +14	Prior to Conditioning vs. 2d after conditioning regiment initiation ↑ 51Cr-EDTA ($p < 0.005$). Prior to Conditioning vs. Day +1 ↑ 51Cr-EDTA ($p < 0.005$). Prior to Conditioning vs. Day +4 ↑ 51Cr-EDTA ($p < 0.0005$). Prior to Conditioning vs. Day +7 ↑ 51Cr-EDTA ($p < 0.0005$). Prior to Conditioning vs. Day +10 ↑ 51Cr-EDTA ($p < 0.0005$).
		Johansson 2007 [108] 38	Preconditioning, Day -1, Day +4, Day +7, Day +14	In patients with GvHD, intestinal permeability increased from baseline to D−1. Subsequently, patients with grade 2–4 GvHD exhibited a further increase from D−1 to Day +4, whereas permeability remained relatively stable in patients with grade 0–1 GvHD. In

				both groups, intestinal permeability remained stable at Day +7 and declined by Day +14, approaching baseline levels.
		Johansson 2001 [109] 17	Preconditioning, Day -1, Day +4, Day +7, Day +14	Baseline vs. Other Samples Reduced-intensity conditioning No statistically significant changes in intestinal permeability were observed at any time point relative to baseline in patients receiving RIC. Myeloablative conditioning In patients receiving myeloablative conditioning, a significant increase in intestinal permeability was observed when samples collected on D−1 (1.1 ± 0.3 vs. 2.6 ± 0.7; $p < 0.005$), Day +4 (1.1 ± 0.3 vs. 3.9 ± 2.6; $p < 0.005$), Day +7 (1.1 ± 0.3 vs. 4.3 ± 3.2; $p < 0.01$), and Day +14 (1.1 ± 0.3 vs. 2.6 ± 1.2; $p < 0.005$) were compared with baseline.
Biomarker-based Methodology	REG3α [1,102]	Wang 2023 [102] 80	Preconditioning Day +7 Day +30	Preconditioning vs. Day +7 ↑ REG3α (values NR; *p* value <0.001). Day +7 vs. Day +30 ↓ REG3α (values NR; *p* value <0.01).
		Rashidi 2018 [1] 95	Preconditioning Day +7 Day +28	Preconditioning vs. Healthy Controls Patients exhibited significantly higher baseline Reg3a levels compared with

				healthy controls (7.07 vs. 4.70; p = 0.006). All Samples Patients exhibited an initial decline from preconditioning to Day +7, followed by recovery through Day +28.
	ST2 [102]	Wang 2023 [102] 80	Preconditioning Day +7 Day +30	Preconditioning vs. Day +7 ↑ ST2 (values NR; *p* value <0.001). Day +7 vs. Day +30 Similar ST2 (values NR; *p* value = 0.58).
	LBP [2,102,103]	Wang 2023 [102] 80	Preconditioning Day +7 Day +30	Preconditioning vs. Day +7 ↑ LBP (values NR; *p* value <0.0001). Day +7 vs. Day +30 ↓ LBP (values NR; *p* value < 0.0001). LBP vs. L:R Ratio There was a significant positive correlation between both markers (r^2 = 0.208; *p* = 0.0014).
		Skaarud 2021 [103] 117†	Baseline (pre-HSCT) Day +21	Baseline vs. Day +21 ↑ Intervention group: 13.794 vs. 18.618; *p* < 0.001. ↑ Control group: 13.389 vs. 18.618; *p* < 0.001.
		Blijlevens 2005 [8]	Day -12 Day -6	Day -12: 18.1 (95% CI 14.1-22.1). Day -6: 16.9 (95% CI 14.0-19.9).

		32	Day 0 Day +7 Day +14 Day +21	Day 0: 27.1 (95% CI 23.2-31.0). Day +7: 53.8 (95% CI 43.0-64.7). Day +14: 36.4 (95% CI 25.1-47.6). Day +21: 27.0 (95% CI 17.6-36.4). Preconditioning (Day -12) vs. Day 0 There was a significant increase in LBP levels at Day 0 ($p < 0.05$). Preconditioning (Day -12) vs. Day +7 There was a significant increase in LBP levels at Day +7 ($p < 0.05$). Preconditioning (Day -12) vs. Day +14 There was a significant increase in LBP levels at Day +14 ($p < 0.05$). Preconditioning (Day -12) vs. Day +21 There was a significant increase in LBP levels at Day +21 ($p < 0.05$).
		Ye 2012 [2] 73	Day -9 Day 0-1 Day +7 Day +14 Day +21 Day +28 Day +56 Day +90-100	All samples Patients with aGvHD Day -9: 7989.0 Day -1: 21657.1 Day +7: 18562.7 Day +14: 21966.4 Day +21: 14924.9 Day +28: 16699.7 Day +56: 12128.8

			At the onset of aGvHD At the time of complete remission.	Day +90-Day +100: 13322.5 Overall, LBP levels increased, peaking on Day +14, and remained above baseline levels until Day +100. Patients without aGvHD Day -9: 1500.0 Day -1: 12271.2 Day +7: 7057.1 Day +14: 9706.7 Day +21: 8570.2 Day +28: 7140.7 Day +56: 6798.8 Day +90-Day +100: 10609.1 Overall, LBP levels increased, peaking on Day -1, and remained above baseline levels until Day +100.
	Occludin [102]	Wang 2023 [102] 80	Preconditioning Day +7 Day +30	Preconditioning vs. Day +7 Occludin (values NR; *p* value not significant). Day +7 vs. Day +30 Occludin (values NR; *p* value not significant).
	Claudin1 [102]	Wang 2023 [102] 80	Preconditioning Day +7 Day +30	Preconditioning vs. Day +7 ↑ Claudin1 (values NR; *p* value <0.01). Day +7 vs. Day +30 Claudin1 (values NR; *p* value < 0.57).

	IAP [102]	Wang 2023 [102] 80	Preconditioning Day +7 Day +30	Preconditioning vs. Day +7 ↓ IAP (values NR; *p* value <0.01). Day +7 vs. Day +30 ↓ IAP (values NR; *p* value < 0.01).
	I-BABP [7]	Derikx 2009 [7] 29	Day -12 Day -6 Day 0 Day +7 Day +14 Day +21	All Samples I-BABP levels decreased significantly over the study period, reaching a nadir at Day +0; however, by Day +21, levels had returned to values comparable to baseline.
	I-FABP [1,7,103]	Skaarud 2021 [103] 117†	Baseline (pre-HSCT) Day +21	Baseline vs. Day +21 ↓ Intervention group: 397 vs. 242pg/ml; *p* = 0.003. ↓ Control group: 473 vs. 204 pg/ml; *p* < 0.001
		Rashidi 2018 [1] 95	Preconditioning Day +7 Day +28	Preconditioning vs. Healthy Controls Patients exhibited significantly higher baseline I-FABP levels compared with healthy controls (1.36 vs. 0.93; p = 0.03). All Samples Patients exhibited an initial decline from preconditioning to Day +7, followed by recovery through Day +28.
		Derikx 2009 [7] 29	Day -12 Day -6 Day 0	All Samples I-FABP levels decreased significantly over the study period, reaching a nadir

			Day +7 Day +14 Day +21	at Day +7; however, by Day +21, levels had returned to values comparable to baseline.
	sCD14 [103]	Skaarud 2021 [103] 117†	Baseline (pre-HSCT) Day +21	Baseline vs. Day +21 Intervention group: 1.651 vs. 21750; $p = 0.437$ Control group: 1820 vs. 1829; $p = 0.651$
	Fecal Zonulin [104]	Tyszka 2022 [104] 32	Day -7 Day +7 Day +14 Day +21	Day -7 vs. Reference values ↑ Zonulin levels (76.45 vs. <30). Day +7 vs. Reference values ↑ Zonulin levels (39.95 vs. <30). Day +14 vs. Reference values ↑ Zonulin levels (43.00 vs. <30). Day +21 vs. Reference values ↑ Zonulin levels (41.45 vs. <30). Day -7 vs. Day +7 ↓ Zonulin levels (76.45 vs. 39.95; $p < 0.001$). Day -7 vs. Day +14 ↓ Zonulin levels (76.45 vs. 43.00; p value NR). Day -7 vs. Day +21 ↓ Zonulin levels (76.45 vs. 41.45; $p < 0.001$).

				L:M Ratio vs. Zonulin There was no significant correlation between L:M Ratio or ΔL:M Ratio and zonulin.
	Beta-defensin-2 [104]	Tyszka 2022 [104] 14	Day -7 Day +7 Day +14 Day +21	Day -7 vs. Refence values ↓ Beta-defensin-2 levels (17.90 vs. <60). Day +7 vs. Refence values ↓ Beta-defensin-2 levels (7.80 vs. <60). Day +14 vs. Refence values ↓ Beta-defensin-2 levels (4.31 vs. <60). Day +21 vs. Refence values ↓ Beta-defensin-2 levels (6.20 vs. <60). Day -7 vs. Day +7 Similar Beta-defensin-2 levels (17.90 vs. 7.80; p = 0.101). Day -7 vs. Day +14 ↓ Beta-defensin-2 levels (17.90 vs. 4.31; p value NR). Day -7 vs. Day +21

				↓ Beta-defensin-2 levels (17.90 vs. 6.20; p = 0.006). L:M Ratio vs. Beta-defensin-2 There was no significant correlation between L:M Ratio or ΔL:M Ratio and beta-defensin-2.
	Calprotectin [104]	Tyszka 2022 [104] 31	Day -7 Day +7 Day +14 Day +21	Day -7 vs. Reference values ↓ Calprotectin levels (15.50 vs. <50). Day +7 vs. Reference values ↓ Calprotectin levels (21.50 vs. <50). Day +14 vs. Reference values ↓ Calprotectin levels (19.30 vs. <50). Day +21 vs. Reference values ↓ Calprotectin levels (16.90 vs. <50). Day -7 vs. Day +7 Similar calprotectin levels (15.50 vs. 21.50; p = 0.374). Day -7 vs. Day +14 Similar calprotectin levels (15.50 vs. 19.30; p value NR). Day -7 vs. Day +21 Similar calprotectin levels (15.50 vs. 16.90; p = 0.451).

				L:M Ratio vs. Calprotectin There was no significant correlation between L:M Ratio or ΔL:M Ratio and calprotectin.
	Citrulline [1,3–8,105]	Pontoppidan 2015 [105] 30	Baseline (7 to 14 days prior to allo-HSCT) Day 0 Day +7 Day +14 Day +21 Day +28	Baseline vs. Day 0 ↓ Citrulline levels (values NR; $p < 0.001$). Baseline vs. Day +7 ↓ Citrulline levels (23 ± 1.5 vs. 7.4 ± 1.0 umol/L; $p < 0.001$). Baseline vs. Day +14 ↓ Citrulline levels (values NR; $p < 0.001$). Baseline vs. Day +21 ↓ Citrulline levels (values NR; $p < 0.05$). Baseline vs. Day +28 ↓ Citrulline levels (values NR; $p < 0.05$).
		Blijlevens 2005 [4] 32	Preconditioning Day +7 Day +21	Preconditioning Glutamine group: 34 (±8). Placebo group: 30 (±7). Day +7 Glutamine group: 11 (±5).

				Placebo group: 10 (±10). Day +21 Glutamine group: 18 (±6). Placebo group: 12 (±3).
		Blijlevens 2004 [6] 32	Day -12 Day -6 Day 0 Day +7 Day +14 Day +21	Day -12: 31.8 (95% CI 28.2-35.3). Day -6: 25.7 (95% CI 21.7-35.3). Day 0: 15.1 (95% CI 12.8-17.4). Day +7: 9.9 (95% CI 7.9-11.8). Day +14: 14.1 (95% CI 11.2-16.9). Day +21: 14.5 (95% CI 12.2-16.8). Preconditioning (Day -12) vs. Day 0 Citrulline levels were significantly decreased at Day 0 compared with baseline ($p < 0.0001$).
		Blijlevens 2005 [8] 32	Day -12 Day -6 Day 0 Day +7 Day +14 Day +21	Day -12: 31.8 (95% CI 28.2-35.3). Day -6: 25.7 (95% CI 21.7-35.3). Day 0: 15.1 (95% CI 12.8-17.4). Day +7: 9.9 (95% CI 7.9-11.8). Day +14: 14.1 (95% CI 11.2-16.9). Day +21: 14.5 (95% CI 12.2-16.8). Preconditioning (Day -12) vs. Day 0 Citrulline levels were significantly decreased at Day 0 compared with baseline ($p < 0.0001$). Preconditioning (Day -12) vs. Day +7

				Citrulline levels were significantly decreased at Day +7 compared with baseline ($p < 0.05$). Preconditioning (Day -12) vs. Day +14 Citrulline levels were significantly decreased at Day +14 compared with baseline ($p < 0.05$). Preconditioning (Day -12) vs. Day +21 Citrulline levels were significantly decreased at Day +21 compared with baseline ($p < 0.05$).
		Lutgens 2005 [5] 10	Daily from Day -6 to Day +1 Every other day until from Day +1 to Day +7 Daily until Day +12 Single sample Day +21	All Samples Overall, citrulline concentrations declined significantly over time ($p < 0.001$), reaching a nadir at Day +5 (mean 8.9; 95% CI 4.4–13.4). Compared with baseline, citrulline levels were significantly lower at all time points, returning to values comparable to baseline by Day +21. Day -6: 25.7 (95% CI 17.1–34.4). Day -5: 21.5 (95% CI 14.4–28.6). Day -4: 18.1 (95% CI 10.3–26.1). Day -3: 18.6 (95% CI 9.5–27.8). Day -2: 15.6 (95% CI 9.9–21.2). Day -1: 14.5 (95% CI 8.4–20.7).

				Day 0: 12.3 (95 CI 8.0–16.5). Day +1: 11.6 (95% CI 6.6-16.6). Day +3: 9.7 (95% CI 5.3–14.1). Day +5: 8.9 (95% CI 4.4–13.4). Day +7: 9.9 (95% CI 5.5–14.2). Day +8: 11.3 (95% CI 6.3–16.3). Day +9: 11.5 (95% 6.1–17.0). Day +10: 12.4 (95% CI 10.5-14.2). Day +11: 15.7 (95% CI 8.6–22.9). Day +12: 13.8 (95% CI 6.1–21.4). Day +21: 20.1 (95% CI 11.1–29.1).
		Rashidi 2018 [1] 95	Preconditioning Day +7 Day +28	Preconditioning vs. Healthy Controls Patients exhibited significantly lower baseline citrulline levels compared with healthy controls (1.88 vs. 10.8; $p < 0.01$). All Samples Patients exhibited an initial decline from preconditioning to Day +7, followed by recovery through Day +28.
		Van der Velden 2010 [3] 86	Three times weekly from hospital admission to Day +20	All Samples Regardless of conditioning regimen, patients exhibited a decrease in citrulline levels during allo-HSCT that remained below baseline at Day +20. Only patients receiving cyclophosphamide and fludarabine conditioning achieved citrulline levels comparable to baseline by Day +20.

		Derikx 2009 [7] 29	Day -12 Day -6 Day 0 Day +7 Day +14 Day +21	All Samples Citrulline levels declined significantly after allo-HSCT, reaching a nadir on Day +7, followed by partial recovery through Day +21, while remaining below baseline levels.
	ZO-1 [106]	Nalle 2019 [106] 9	Biopsy was collected at least 2 weeks after allo-HSCT	Patient Sample vs. Controls Patients with GvHD exhibited similar distribution of ZO-1 when compared to healthy controls subjects.
	Actin [106]	Nalle 2019 [106] 9	Biopsy was collected at least 2 weeks after allo-HSCT	Patient Sample vs. Controls Patients with GvHD exhibited similar distribution of actin when compared to healthy controls subjects.
	MLCK [106]	Nalle 2019 [106] 9	Biopsy was collected at least 2 weeks after allo-HSCT	Patient Sample vs. Controls Patients with GvHD exhibited increased MLCK expression when compared to healthy controls subject ($p < 0.05$).

51Cr-EDTA Absorption: Chromium-51–labeled ethylenediaminetetraacetic acid absorption; Allo-HSCT: Allogeneic hematopoietic stem cell transplantation; D: day; I-FABP: Intestinal fatty acid binding protein; LBP: LPS binding protein; ST2: Suppression of tumorigenicity-2 protein; sCD14: soluble cluster of differentiation; MLCK: long myosin light chain kinase; REG3: Regenerating family member-3 alpha; Not Reported; ZO: Zonula Occludens; †: this study investigated gut barrier function in response to a dietary intervention

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5.3. Manuscritos Originais

5.3.1. Manuscrito I – Eventos de Dominância Intestinal no Alo-TMO

Journal of *Clinical Medicine*

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Article

A Distinct Intestinal Domination Fingerprint in Patients Undergoing Allo-HSCT: Dynamics, Predictors and Implications on Clinical Outcomes

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Abstract

Background: Although *Enterococcus* domination has been extensively evaluated in the context of allogeneic hematopoietic stem cell transplantation (allo-HSCT), the prevalence and clinical implications of other dominant genera remain poorly understood. **Objective:** In this study, we sought to determine the dynamics, predictors and clinical implications of intestinal domination in Brazilian patients undergoing allo-HSCT. **Methods:** In a prospective study of four Brazilian centers, fecal specimens were collected longitudinally prior to allo-HSCT until six months post-transplantation. To identify intestinal domination, we performed 16S rRNA gene sequencing using the Illumina platform. We then evaluated the impact of intestinal domination on overall survival and acute Graft-versus-Host-Disease (aGvHD) incidence. Finally, to identify predictors of intestinal domination, we performed a logistic regression model. **Results:** A total of 192 fecal specimens were collected from 69 patients. No significant changes in alpha or beta diversity were observed over the course of allo-HSCT. Among the 192 specimens, 131 (68%) presented intestinal domination. The top four dominant genera were *Bacteroides*, *Akkermansia*, *Phascolarctobacterium*, and *Escherichia-Shigella*. No significant associations were found between domination by these genera and either overall survival or aGvHD incidence. Furthermore, no patient-level characteristics, including age, sex, underlying disease, conditioning regimen, or stem cell source, reliably predicted intestinal domination. **Conclusions:** Our findings reveal a unique intestinal domination fingerprint in Brazilian patients and highlight the importance of geographic context in interpreting microbiota–outcome associations in allo-HSCT settings.

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Keywords: hematopoietic stem cell transplantation; Graft-versus-Host-Disease; intestinal microbiome; intestinal domination; clinical outcomes

1. Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a curative therapy for several malignant and non-malignant diseases [1–6]. Despite allo-HSCT's potential benefits, life-threatening complications such as neutropenic fever, relapse and acute Graft-versus-Host-Disease (aGvHD) remain major barriers to successful outcomes [1–6]. The clinical success of allo-HSCT relies on the identification and mitigation of key variables with prognostic significance. Among these prognostic variables that can shape the course of the allo-HSCT, a key player seems to be the intestinal microbiota [7–9]. The intestinal microbiota plays a multifaceted role in human physiology, contributing to the preservation of the gut barrier integrity, producing key metabolites to maintain gut homeostasis, and shaping immune responses through complex crosstalk [10]. Through these mechanisms, the intestinal microbiota can shape the development of clinical outcomes over the allo-HSCT. For instance, aGvHD, which is the leading cause of non-relapse mortality, has been linked to specific patterns of intestinal microbiota disruption ("intestinal dysbiosis") [11–15]. A decreased intestinal diversity (an index that measures intestinal microbiota variety [richness] and balance [evenness]) over the engraftment period has been associated with an approximately eight-fold increase in the risk of aGvHD [12]. Another intestinal dysbiosis feature frequently reported is the decreased abundance of SCFA (short-chain-fatty-acid)-producing bacteria, which has been associated with infections, transplant-related mortality, and overall survival [16–18]. These and other studies outline the impact of specific intestinal dysbiosis features in the context of allo-HSCT.

In patients undergoing allo-HSCT, another key feature of intestinal dysbiosis is the expansion of a single microbiota genus resulting in intestinal domination. Intestinal domination is a frequent dysbiosis feature in allo-HSCT, occurring in 28% to 80% of patients [19–24]. Although intestinal domination is common, the specific genus driving this event may vary across studies. For example, while *Enterococcus* was the most common dominant genus in the microbiota of patients from four different centers, *Streptococcus* and *Bacteroides* were predominant in other allo-HSCT studies [19,21,24]. Thus, although intestinal domination is consistently present in allo-HSCT studies, distinct domination fingerprints may emerge across different cohorts.

Across different cohorts, understanding how these distinct domination fingerprints may have prognostic significance is a key step to optimizing stratification and intervention strategies. Nevertheless, the available evidence has largely been focused on *Enterococcus* domination [20–24]. For instance, *Enterococcus* domination has been linked to significantly reduced overall survival, GvHD severity, and increased risk of bloodstream infections (BSI) [21,23,24]. However, how other dominant genera may impact clinical outcomes remains poorly understood.

Therefore, in this study, we sought to provide a comprehensive overview of the dynamics, predictors, and clinical implications of intestinal domination events in a cohort of Brazilian patients undergoing allo-HSCT. As shown in the subsequent sections of this manuscript, we identified a unique domination fingerprint in our cohort and conclude that the prognostic significance of intestinal domination during allo-HSCT may vary according to the dominant genera and the population being evaluated.

2. Patients, Material and Methods

2.1. Study Design, Ethical Aspects and Sample Collections

This is an ongoing, observational, multicenter, prospective, cohort study of patients undergoing allo-HSCT, which was approved by the Research Ethics Committee of Sao Paulo State University (process number 5.138.190/2021) and conducted according to the guidelines of the Declaration of Helsinki. The inclusion criteria were patients $\geq$ 12 years old undergoing allo-HSCT who provided at least one stool sample during the procedure and had clinical data available in REDCap. The exclusion criteria were: (1) cord blood transplant recipients, (2) patients who withdraw from the study, and (3) patients who did not provide stool samples over the 6-month follow-up. Antibiotic administration, nutritional status or presence of comorbidity was not considered as exclusion criteria. All participants signed an informed consent form at the beginning of the study. For patients <18 years old, both the patient and their legal representative agreed to participate in the study and signed consent forms.

Stool samples were collected at four different transplant centers (Hospital de Base of the Fundação Faculdade Regional de Medicina [HB-FUNFARME], Hospital Amaral Carvalho [HAC], Hospital de Cancer de Barretos [HCB] and Hospital Beneficência Portuguesa de São Paulo [BP]) and stored at −80 °C. Stool samples were collected longitudinally at 7 time points: prior to conditioning regimen (D−7), at the day of stem cell infusion (D0), 30 days after stem cell infusion (D+30), D+60, D+90, D+180, and at acute GvHD diagnosis. Although all efforts were made to collect the specimens at these exact time points, some variability occurred due to the timing and frequency of bowel movement patterns. Additionally, some samples were not collected from patients who were discharged from the original institution to continue follow-up in their hometown or, in some cases, when the patient was critically ill.

2.2. Intestinal Microbiota 16S Sequencing and Bioinformatics Pipeline

DNA was extracted from 200 mg of fecal samples by using QIAamp Fast DNA Stool Mini Kit (Qiagen, CA, USA), according to the manufacturer's protocol. DNA was then quantified by Qubit dsDNA HS Assay Kit (Thermofisher, Waltham, MA, USA). The 16S rRNA V3-V4 regions were then amplified in a two-step PCR protocol using the following predetermined primers—341F: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG, and 805R 5′- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. PCR reactions used 2X Platinum SuperFi II PCR Master Mix (Thermofisher) and involved a denaturation at 98 °C for 30 s followed by 30 cycles of: 98 °C for 10 s, 55 °C for 10 s, and 72 °C for 30 s. Sequencing was done in the Illumina NextSeq 1000 platform (San Diego, CA, USA). A total of 45,590,208 reads were generated (average ~228,000 per sample) and underwent adapter removal using the Cutadapt v2.6 [25]. Then, the reads underwent quality filtering, denoising, chimera removal and amplicon sequence variant (ASV) inference using DADA2 v.1.26.0 [26]. A total of 17,934,483 reads (average ~89,700 per sample) passed all filtering and were used in downstream analyses. Taxonomic classification was performed using the Classify-sklearn naive Bayes classifier as implemented in Qiime2 (q2-feature-classifier plugin), which was trained on the SILVA SSU v138.1 NR 99 database [27,28]. The relative abundance of different ASVs in each sample was analyzed in Qiime2 and plotted with the taxa plugin (barplot function). Alpha and beta diversity indexes as well as statistical tests for significant differences across experimental groups were computed using the Qiime2 diversity plugin. The normalization method used in the study was rarefaction to 50,000 reads per sample (Qiime2 diversity plugin, alpha-rarefaction function; see Supplementary Figure S1). Tables were developed to compute the abundance of reads assigned to each ASV, which were then aggregated

to the genus level for analyses. The metadata from this study have been submitted to the NCBI Sequence Read Archive (BioProject PRJNA1357096).

2.3. Statistical Analysis

Intestinal domination was defined as the relative abundance $\geq$ 30% of any specific genera within each stool sample [19,21,23,24,29]. This threshold was selected to align with prior allo-HSCT studies and ensure consistency with global definitions of intestinal domination. This analysis was focused on genera because this is generally the most specific level at which 16S sequencing can provide reliable classification [17]. We first generated descriptive statistics separated by intestinal domination status. Tests of associations between intestinal domination status and variables were examined by chi-square or Fisher's exact test for categorical variables and Wilcoxon rank-sum test for continuous variables. Consistent with prior studies evaluating intestinal domination, the outcomes of interest were overall survival and cumulative incidence of aGvHD and severe aGvHD (grade 2–4) [17,21,24,30]. Overall survival was analyzed using the Kaplan–Meier methodology and survival curves were compared using the log-rank test. The time to event was calculated from D0 (day of stem cell infusion) to the date of death by any cause, aGvHD or last follow-up. Acute GvHD was diagnosed clinically, confirmed pathologically by biopsy, whenever possible, and graded per the MAGIC criteria [30]. Similar to prior studies, the onset of aGvHD was determined based on the onset of symptoms (clinical suspicion) [15]. A Cox regression analysis was used to evaluate the association between intestinal domination and aGvHD. A multivariate Cox regression analysis was performed to evaluate the association between intestinal domination and overall survival, as well as the cumulative incidence of GvHD, adjusting for potential confounders (age, sex, center, underlying diagnosis, conditioning regimen, donor sex, stem cell source, and donor type). This multivariate analysis was not conducted for severe GvHD due to the relatively low number of patients. Univariate logistic regression models were used to identify predictors of intestinal domination (see Supplementary Methods for details). A sensitivity analysis was performed specifically for overall survival to identify the optimal cutoff value for defining intestinal domination (expressed as relative abundance, %). For each potential threshold, samples were classified into "dominant" and "non-dominant" groups, which were then compared using Cox proportional hazards regression. A post hoc power analysis using the survivalpwr package in R was performed to evaluate whether the small sample size may have contributed to the significant association observed in our analysis, potentially indicating instability of the effect estimate [31]. All analyses were performed using the software R. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. Diversity Metrics and Prevalence of Intestinal Domination

During the study period, 69 patients undergoing allo-HSCT provided 192 stool samples. The proportion of samples at each time point is shown in Supplementary Table S1. No significant changes in alpha or beta diversity were observed over the course of allo-HSCT (see Figure 1).

At the patient level, the prevalence of intestinal domination at any time point was 78.2% (n = 54/69). Among the 192 stool samples, 68.2% (n = 131/192) exhibited an intestinal domination event at any time point. The prevalence of intestinal domination at each time point is shown in Figure 2. The lowest prevalence occurred in samples collected prior to the conditioning regimen (41.6%; n = 20/48). The highest prevalence occurred in samples collected 60 days after allo-HSCT (96.6%; n = 29/30). At the time of aGvHD diagnosis, 70% of patients (n = 7/10) presented intestinal domination by a single genus. Additionally,

among the 131 samples with intestinal domination, 12.9% (n = 17/131) had concurrent domination by two distinct genera (see Supplementary Table S2).

The specific genera responsible for these intestinal domination events are shown in Figure 3. Overall, most of these events resulted from the expansion of four genera: (1) *Bacteroides* (n = 46), (2) *Akkermansia* (n = 19), (3) *Phascolarctobacterium* (n = 16), and (4) *Escherichia–Shigella* (n = 14; see Figure 2). Interestingly, only one sample (0.5%) showed *Enterococcus* domination (see Supplementary Table S3).

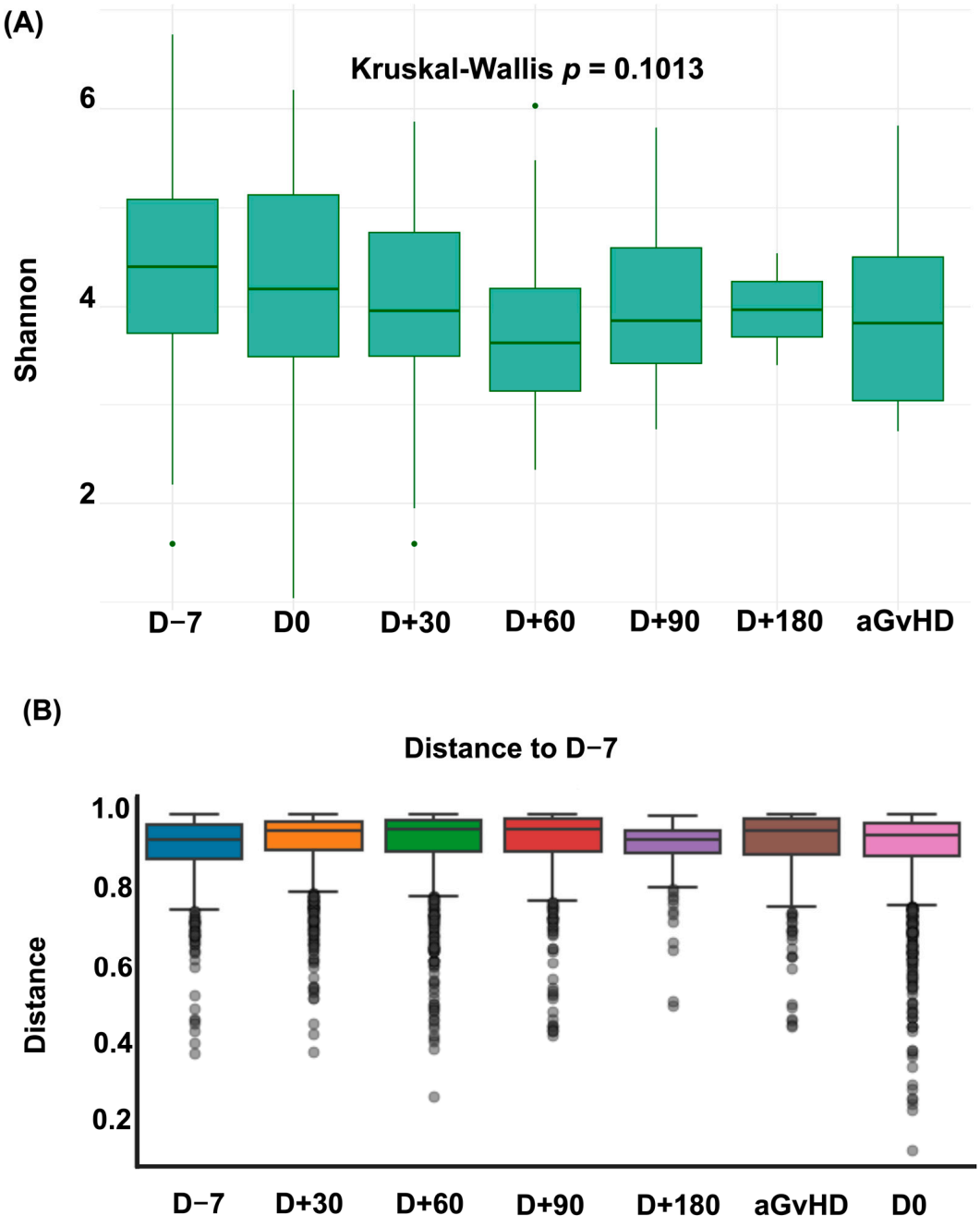


Figure 1. Dynamics of Alpha and Beta Diversity over the allo-HSCT. (**A**) Shannon Index over the allo-HSCT. (**B**) Bray–Curtis dissimilarity relative to pre-conditioning sample (D − 7). Each box represents the distribution of dissimilarities across patients at each time point. Higher distances indicate greater shifts in microbiota composition relative to baseline. aGvHD = acute graft versus host disease; D = Day.

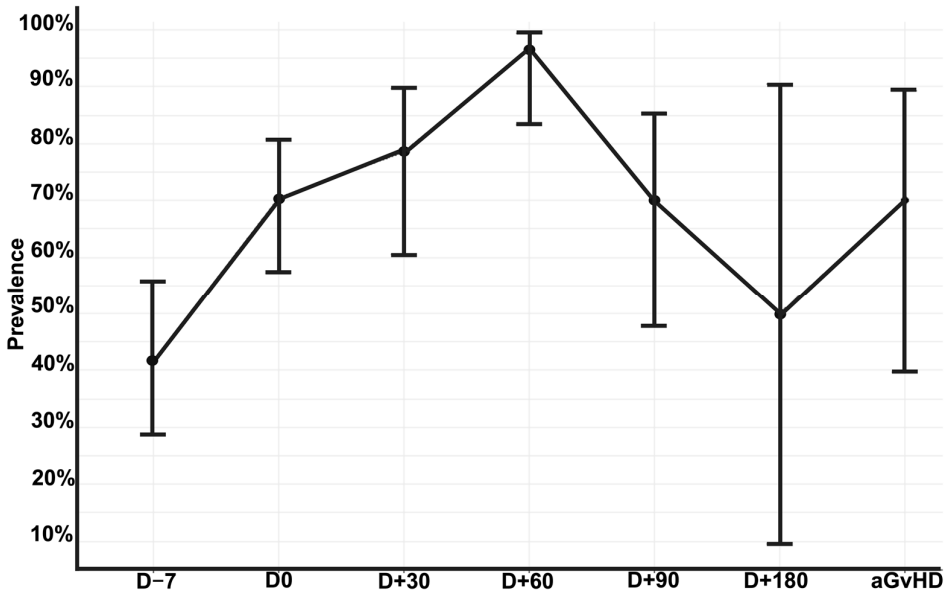


Figure 2. Prevalence of intestinal domination by any genus over the allo-HSCT. Error bars representing 95% confidence interval calculated using the Wilson score method for proportions to account for small sample sizes at late time points. Allo-HSCT = Allogeneic hematopoietic stem cell transplantation. aGvHD = acute Graft-versus-Host Disease; D = Day. Edited with BioRender.com, Soares Ferreira Junior A. (2025) https://BioRender.com/zd0vcep (accessed on 3 November 2025).

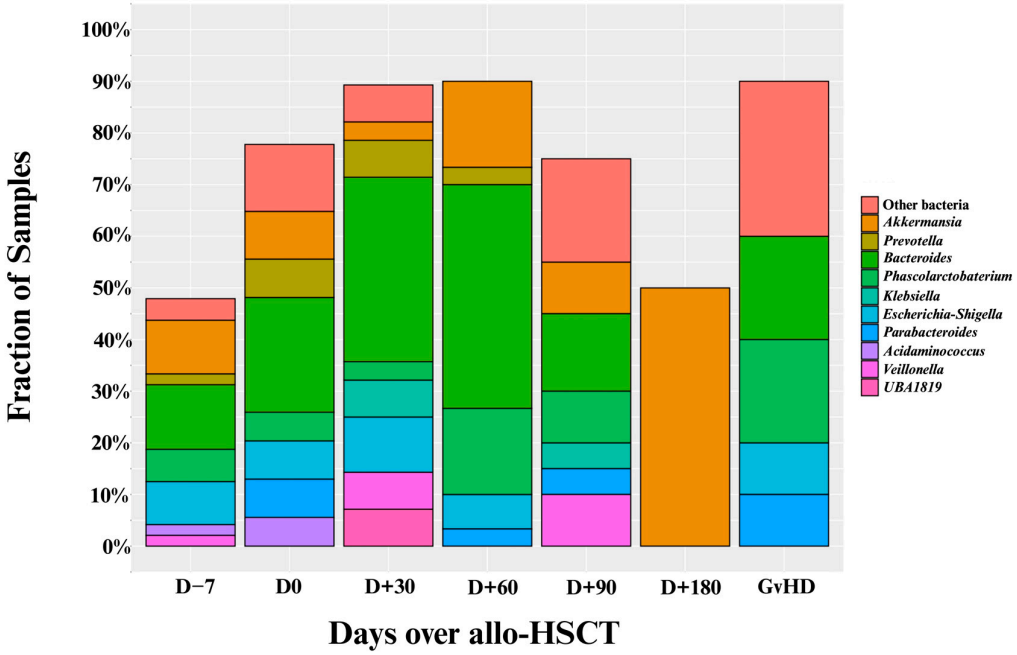


Figure 3. Proportion of intestinal domination events by specific genera over the allo-HSCT. Allo-HSCT = Allogeneic hematopoietic stem cell transplantation. Edited with BioRender.com, Soares Ferreira Junior A. (2025) https://BioRender.com/xzrbtuf (accessed on 3 November 2025).

3.2. Patient Characteristics by Intestinal Domination Status

Patient demographic information classified by intestinal domination status is shown in Table 1. No statistically significant differences were found for any of the demographic characteristics (age, weight, height, and sex). Additionally, no statistically significant differences were found for any allo-HSCT-related variable (prior allo-HSCT, stem cell source, donor type, and conditioning regimen).

Table 1. Baseline Demographic Characteristics by Intestinal Domination Status.

Variable	No-Intestinal Domination (*n* = 15)	Yes-Intestinal Domination (*n* = 54)	Total (*N* = 69)	*p*-Value
Age (years)				0.6
Mean (SD)	44 (19)	41 (15)	40 (16)	
Median (IQR)	40 (27–68)	42 (31–51)	40 (28–49)	
Range	18–74	12–71	12–73	
Weight (kg)				0.12
Mean (SD)	79 (16)	72 (17)	74 (17)	
Median (IQR)	79 (68–87)	68 (62–82)	74 (64–82)	
Range	48–110	43–130	43–130	
Height (cm)				0.091
Mean (SD)	170 (10)	166 (10)	167 (10)	
Median (IQR)	173 (160–178)	164 (158–174)	165 (160–175)	
Range	150–185	146–189	146–189	
Center				0.2
HB-FUNFARME	4 (27%)	5 (9.3%)	9 (13%)	
HAC	4 (27%)	18 (33%)	22 (32%)	
HCB	0 (0%)	8 (15%)	8 (12%)	
BP	7 (47%)	23 (43%)	30 (43%)	
Sex				0.2
Male	9 (60%)	22 (41%)	31 (45%)	
Female	6 (40%)	32 (59%)	38 (55%)	
Prior Allo-HSCT	0 (0%)	3 (6.4%)	3 (5.1%)	>0.9
Primary Disease				0.4
Acute Myeloid Leukemia	6 (40%)	23 (43%)	29 (42%)	
Acute Lymphoid Leukemia	1 (6.7%)	15 (28%)	16 (23%)	
Chronic Myeloid Leukemia	1 (6.7%)	2 (3.7%)	3 (4.3%)	
Hodgkin's Lymphoma	1 (6.7%)	1 (1.9%)	2 (2.9%)	
Non-Hodgkin's Lymphoma	0 (0%)	1 (1.9%)	1 (1.4%)	
Aplastic Anemia	2 (13%)	5 (9.3%)	7 (10%)	
Sickle Cell Disease	1 (6.7%)	2 (3.7%)	3 (4.3%)	
Other	3 (20%)	5 (9.3%)	8 (12%)	
Stem Cell Source				0.4
Peripheral Blood	9 (60%)	39 (72%)	48 (70%)	
Bone Marrow	6 (40%)	15 (28%)	21 (30%)	
Donor Type				0.11
Matched Related	3 (20%)	17 (31%)	20 (29%)	

Table 1. *Cont.*

Variable	No-Intestinal Domination (*n* = 15)	Yes-Intestinal Domination (*n* = 54)	Total (*N* = 69)	*p*-Value
Matched Unrelated	4 (27%)	3 (5.6%)	7 (10%)	
Mismatched Related	0 (0%)	3 (5.6%)	3 (4.3%)	
Haploidentical	7 (47%)	30 (56%)	37 (54%)	
Mismatched Unrelated	1 (6.7%)	1 (1.9%)	2 (2.9%)	
Donor Sex				0.4
Male	7 (47%)	33 (61%)	40 (58%)	
Female	8 (53%)	21 (39%)	29 (42%)	
Intensity of Conditioning Regimen				0.8
Ablative	4 (27%)	21 (39%)	25 (36%)	
Reduced Intensity	7 (47%)	20 (37%)	27 (39%)	
Nonmyeloablative	4 (27%)	12 (22%)	16 (23%)	
TBI-Conditioning Regimen	6 (40%)	29 (54%)	35 (51%)	0.3

Allo-HSCT = Allogeneic hematopoietic stem cell transplant; BP = Hospital Beneficencia Portuguesa de Sao Paulo; kg = kilograms; cm = centimeters; SD = Standard deviation; IQR = Interquartile range; TBI = Total body irradiation; HB-FUNFARME = Hospital de Base of Fundacao Faculdade Regional de Medicina; HAC = Hospital Amaral Carvalho; HCB = Hospital de Cancer de Barretos.

3.3. Analysis of Clinical Outcomes

Our analysis identified no significant association between overall survival and intestinal domination by the four most frequently identified genera—*Bacteroides* ($p = 0.84$), *Akkermansia* ($p = 0.47$), *Phascolarctobacterium* ($p = 0.4$), and *Escherichia-Shigella* ($p = 0.98$; see Figure 4A–D). Although univariate analysis revealed a significant association between *Phascolarctobacterium* domination and cumulative incidence of aGvHD (HR 2.39 [1.08–5.31]; $p = 0.032$), this was no longer significant after adjusting for confounders (age, sex, center, underlying diagnosis, conditioning regimen, donor sex, stem cell source, and donor type) in a multivariate analysis (HR 1.75 [0.73–4.20]; $p = 0.20$). Also, post hoc power analysis showed 67% power to detect the observed effect size (HR = 2.39) for aGvHD, indicating that the significant association identified in our study may be unstable due to the small sample size and should be interpreted with caution. For the other three genera, no significant associations were found between domination and cumulative incidence of aGvHD (*Bacteroides:* HR = 0.81 [0.40–1.63]; $p = 0.6$; *Akkermansia*: HR = 1.42 [0.59–3.43]; $p = 0.4$; and *Escherichia-Shigella:* HR = 1.94 [0.91–4.17]; $p = 0.088$; see Figure 5A–D). Finally, no significant association was observed between domination by any of the four genera and the cumulative incidence of severe aGvHD—*Bacteroides*: HR = 1.18 [0.44–3.15]; $p = 0.7$; *Akkermansia*: HR = 0.54 [0.16–1.89]; $p = 0.3$; *Phascolarctobacterium:* HR = 0.64 [0.18–2.26]; $p = 0.5$; and *Escherichia-Shigella*: HR = 1.66 [0.54–5.14]; $p = 0.4$ (see Supplementary Figure S2A–D). Multivariate analyses assessing the associations between intestinal domination and overall survival, as well as the cumulative incidence of GvHD, are presented in Supplementary Table S4. The sensitivity analysis performed to identify the optimal cutoff value for defining intestinal domination corroborated the 30% threshold for *Phascolarctobacterium*, and are shown in Supplementary Figure S3A–D.

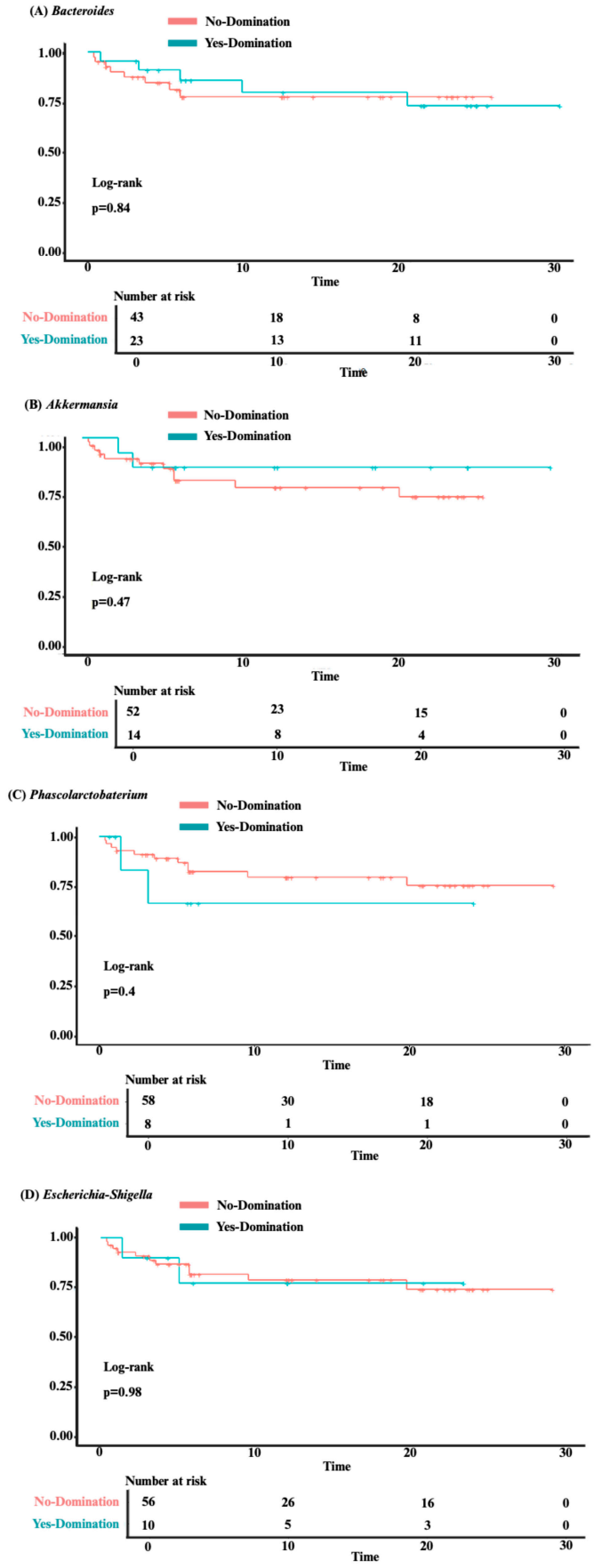


Figure 4. The impact of intestinal domination on overall survival. (**A**) *Bacteroides*. (**B**) *Akkermansia*. (**C**) *Phascolarctobacterium*. (**D**) *Escherichia-Shigella*.

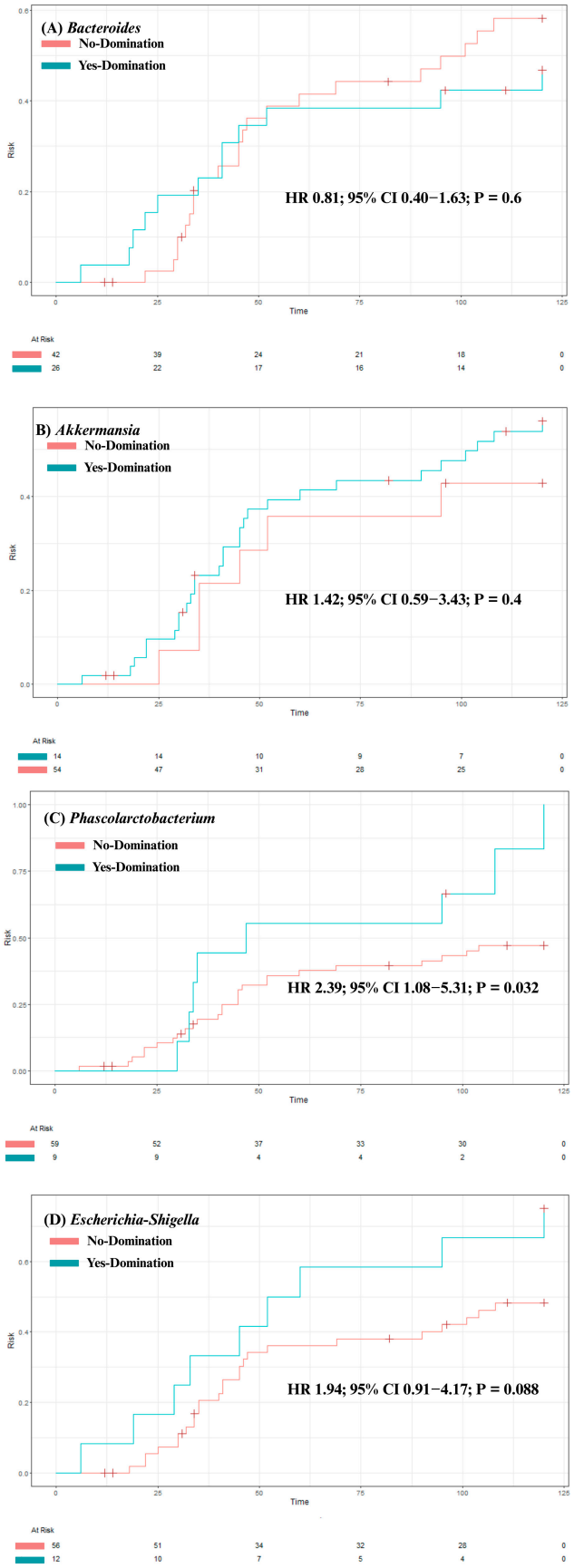


Figure 5. The impact of intestinal domination on the cumulative incidence of aGvHD. (**A**) *Bacteroides*. (**B**) *Akkermansia*. (**C**) *Phascolarctobacterium*. (**D**) *Escherichia-Shigella*. HR = Hazard Ratio. CI = Confidence interval.

3.4. Predictors of Intestinal Domination

The univariate logistic regression model of predictors of intestinal domination by any genera and by *Bacteroides*, *Akkermansia*, *Phascolarctobacterium*, and *Escherichia-Shigella* is shown in Table 2. None of the variables were associated with intestinal domination. Given the absence of statistically significant variables in the univariable model, a multivariable model was not performed.

Table 2. Univariable Logistic Regression Model of Predictors of Intestinal Domination.

	Any Genus		***Bacteroides***		***Akkermansia***		***Phascolarctobacterium***		***Escherichia-Shigella***	
	OR (95%CI)	***p* Value**	**OR (95%CI)**	***p* Value**	**OR (95%CI)**	***p* Value**	**OR (95%CI)**	***p* Value**	**OR (95%CI)**	***p* Value**
Age	0.99 (0.95–1.02)	0.46	0.99 (0.96–1.02)	0.67	0.99 (0.96–1.03)	0.73	1.03 (0.99–1.08)	0.18	1.00 (0.96–1.04)	0.88
BMI	0.98 (0.88–1.09)	0.69	1.01 (0.92–1.11)	0.81	0.99 (0.89–1.11)	0.90	1.05 (0.93–1.20)	0.41	0.94 (0.82–1.06)	0.32
Sex										
Female	-	-	-	-	-	-	-	-	-	-
Male	0.46 (0.14–1.45)	0.19	0.65 (0.24–1.75)	0.40	0.41 (0.10–1.41)	0.18	0.31 (0.04–1.39)	0.16	0.85 (0.23–2.99)	0.80
Conditioning regimen										
Reduced intensity	-	-	-	-	-	-	-	-	-	-
Myeloablative	1.75 (0.46–7.53)	0.42	1.20 (0.39–3.69)	0.75	2.33 (0.61–10.1)	0.23	1.14 (0.19–6.70)	0.88	1.14 (0.24–5.39)	0.86
Non-myeloablative	1.00 (0.25–4.47)	>0.99	1.08 (0.29–3.85)	0.91	1.38 (0.24–7.24)	0.70	1.92 (0.32–11.7)	0.46	2.00 (0.41–9.87)	0.38
TBI-Conditioning Regimen	1.74 (0.55–5.84)	0.35	1.57 (0.59–4.26)	0.37	1.38 (0.43–4.70)	0.59	1.25 (0.30–5.48)	0.76	0.97 (0.27–3.43)	0.96
Stem cell source										
Peripheral	-	-	-	-	-	-	-	-	-	-
Bone marrow	0.58 (0.18–1.98)	0.37	1.03 (0.35–2.93)	0.96	1.35 (0.37–4.58)	0.63	0.25 (0.01–1.50)	0.21	1.83 (0.48–6.60)	0.36
Underlying diagnosis										
Acute leukemia	-	-	-	-	-	-	-	-	-	-
Others	0.40 (0.12–1.33)	0.13	0.76 (0.25–2.20)	0.62	0.89 (0.22–3.10)	0.87	0.25 (0.01–1.50)	0.21	1.18 (0.28–4.29)	0.81

BMI = Body mass index; CI = Confidence interval; OR = Odds ratio; TBI = Total body irradiation.

4. Discussion

In this multicenter, prospective, observational study, we outlined the dynamics, predictors, and clinical implications of intestinal domination in Brazilian patients undergoing allo-HSCT. We first found that intestinal domination is a prevalent dysbiosis fingerprint occurring in 78.2% of patients. Then, we identified a unique pattern of domination in Brazilian patients characterized by an extremely low prevalence of *Enterococcus* domination (only 1.4% of patients). In this fingerprint, most intestinal domination events were driven by the expansion of *Bacteroides*, *Akkermansia*, *Phascolarctobacterium*, and *Escherichia-Shigella.* Although this domination fingerprint was frequent in our cohort, it did not appear to significantly impact key clinical outcomes such as overall survival and aGvHD incidence. Finally, none of the patient or allo-HSCT-related characteristics included in our analysis predicted intestinal domination.

In our analysis, the most interesting finding is that although intestinal domination occurred in 78.2% of patients, this was rarely driven by *Enterococcus* expansion (only one patient). This extremely low prevalence of *Enterococcus* domination (1.4%) differs from the

current literature. In prior studies examining intestinal domination in patients undergoing allo-HSCT, *Enterococcus* has been identified as a key driver of domination events, occurring in 36% to 65% of patients [9,19,21–24]. On the higher end of the spectrum of reported *Enterococcus* domination is a 65% domination prevalence reported in a study including 1325 patients with 9049 stool specimens [21]. In this study, the genus *Enterococcus* was the most commonly observed to dominate the microbiota in patients from the four different participating centers [21]. On the lower end of the spectrum is a 36% *Enterococcus* domination prevalence occurring in a study including 98 patients with 681 stool specimens [24]. In this study, the median time between transplant date and first stool sample with *Enterococcus* domination was 22 days (IQR 6.75–84.25) [24]. It is important to note, however, that each of these studies used distinct sampling time points, which may hinder an accurate comparison across studies [9,19,21–24]. Also, antibiotic use, a known disruptor of intestinal domination, was not collected across all centers and, therefore, excluded from this analysis. This represents a key limitation, as center-specific antibiotic practices (e.g., levofloxacin prophylaxis; see Supplementary Table S5) may explain the low *Enterococcus* prevalence identified in our study. Nevertheless, compared to these studies, our findings suggest that *Enterococcus* may not be a key genus driving intestinal domination in Brazilian patients. Therefore, future studies should investigate which other genera are driving intestinal domination in this cohort.

Our findings identified that important genera driving intestinal domination in Brazilian patients are *Bacteroides*, *Akkermansia*, *Phascolarctobacterium*, and *Escherichia-Shigella*. In our cohort, these four genera were responsible for most of the domination events. While *Bacteroides* and *Akkermansia* have been reported as dominant genera in prior studies, the high frequency of *Phascolarctobacterium* and *Escherichia-Shigella* domination is not well demonstrated in the prior literature [9,19–24]. The dynamics of intestinal domination was evaluated in 100 patients with 603 stool specimens [19]. This study demonstrated an interesting compositional shift [19]. While *Bacteroides* was the most common dominant genus before conditioning, at the time of engraftment, intestinal domination occurred predominantly due to pathogenic genera such as *Enterococcus*, *Klebsiella* and *Escherichia-Shigella* [19]. In another study including 98 patients undergoing allo-HSCT, 681 stool specimens were analyzed [24]. The most frequent genera responsible for intestinal domination, in decreasing order, were as follows: (1) *Streptococcus* (42%), (2) *Enterococcus* (36%), and (3) *Bacteroides* (38%) [24]. Other important dominant genera reported in this study were *Akkermansia*, *Blautia* and *Lactobacillus*, each of which was associated with intestinal domination in approximately 28% of patients. A similar profile was also identified in another study including 94 patients with 439 stool specimens [23]. In this study, *Enterococcus* was the most frequent dominating genus (40%), followed by *Streptococcus* (37%) [23]. When compared to these previous studies, our findings suggest that Brazilian patients undergoing allo-HSCT may exhibit a unique intestinal domination fingerprint.

This unique intestinal domination fingerprint likely reflects a combination of factors that modulate the intestinal microbiota during allo-HSCT. First, it may be partly explained by distinct dietary habits in Brazilian patients (e.g., high intake of fiber, cereals, rice and yogurt) [19,32,33]. High fiber consumption, for instance, has been associated with greater intestinal microbial diversity and increased production of SCFAs [33]. Conversely, unhealthy dietary patterns characterized by high sugar or fat intake have been linked to shifts in microbial composition and decreased intestinal diversity [34]. Second, this fingerprint may result from center-specific antibiotic practices and distinct *Enterococcus* resistance and colonization profiles observed across countries [22,23,35]. Finally, the persistence of *Bacteroides* domination (a common feature in healthy individuals) during allo-HSCT, may also suggest a more resilient microbiota in our cohort [24,33,36,37]. Microbiota resilience refers

to the ability of the microbial community to rapidly return to its original state despite major perturbations that may occur during allo-HSCT (e.g., antibiotic exposure, conditioning regimens, infections, or dietary changes) [33,34]. This hypothesis is further supported by our longitudinal analysis of intestinal diversity over the allo-HSCT, which showed no significant differences between baseline and post-allo-HSCT samples (see Figure 1). Therefore, multiple factors may contribute to this distinctive intestinal domination fingerprint observed in Brazilian patients, and future studies incorporating detailed dietary, antibiotic, and functional experiments assessing microbiota resilience are warranted.

Whether this intestinal domination fingerprint identified in our analysis has prognostic significance was the next question we tried to answer in this study. In our attempt to evaluate the prognostic significance, we evaluated overall survival and aGvHD, which are key clinical outcomes that have been linked with *Enterococcus* domination [20,21,23,24]. For example, in the aforementioned study including 1,325 patients undergoing allo-HSCT, *Enterococcus* domination was associated with an approximately two-fold decrease in overall survival (HR 1.97; 95% CI 1.45–2.66; $p < 0.001$) [21]. This finding remained significant in a multivariate analysis adjusted for graft source, age, conditioning intensity, gender, and underlying disease (HR 2.06; 95% CI 1.50–2.82; $p < 0.0001$) [21]. In this same study, *Enterococcus* domination was also significantly associated with GvHD severity in both univariate (HR 1.44; 95% CI 1.10–1.88; $p < 0.01$) and multivariate (HR 1.32; 95% CI 1.00–1.75; $p < 0.05$) analyses [21]. It is worth noting that few studies in the literature have specifically analyzed the clinical implications of intestinal domination by some of the genera identified in our cohort. *Bacteroides* and *Akkermansia* domination, for example, was not significantly associated with overall survival ($p = 0.08$ and $p = 0.14$) in a prior study with 98 patients [24]. This is further corroborated by our findings, demonstrating that intestinal domination by *Bacteroides*, *Akkermansia*, *Phascolarctobacterium* and *Escherichia-Shigella* may not impact clinical outcomes. Thus, our findings suggest that the prognostic significance of intestinal domination may vary according to the dominant genera and the population being evaluated.

Although evidence directly addressing the prognostic impact of domination by these genera is lacking, prior studies have reported associations between their relative abundances and key clinical outcomes during allo-HSCT [4,24,38–40]. Some of these genera have been associated with aGvHD, overall survival, and relapse-free survival [38–40]. In a previous allo-HSCT study including 71 patients, the relative abundance of *Bacteroides* was significantly lower in patients with aGvHD compared to those without ($p < 0.05$) [38]. In that same study, higher *Bacteroides* abundance was also associated with a reduced incidence of aGvHD ($p = 0.0043$), and improved aGvHD-free/relapse-free survival ($p = 0.021$) [38]. In another study including 37 patients with aGvHD, significantly higher abundances of *Escherichia–Shigella* and *Enterococcus* were observed compared to healthy controls. Additionally, patients with steroid-refractory aGvHD showed significantly reduced *Bacteroides* abundance compared to steroid-responsive patients ($p = 0.007$). Decreased *Akkermansia* abundance was also associated with aGvHD in another study including 150 patients ($p < 0.05$) [4]. Although less explored in the allo-HSCT context, prior studies have highlighted *Phascolarctobacterium* as a key genus associated with diseases such as diabetes through SCFA production [41–43]. In our analysis, although *Phascolarctobacterium* was significantly associated with aGvHD in the univariate analysis, this association did not remain significant in the multivariate model. Taken together, these studies suggest that the genera identified in our cohort may have prognostic relevance when evaluated as continuous measures of relative abundance. The lack of significant associations in our analysis may reflect (1) limited statistical power, and (2) the inherent loss of information caused by categorizing continuous microbial abundances into binary domination events.

Therefore, future large prospective studies are needed to validate these findings and to strengthen the evidence surrounding the prognostic significance of this unique fingerprint.

Given the potential prognostic significance of intestinal domination, the final planned analysis was to identify predictors of intestinal domination in our cohort. Our findings demonstrated that no patient-level characteristics, including age, sex, underlying disease, conditioning regimen, or stem cell source, could reliably predict intestinal domination, although antibiotic use (a known microbiota disruptor) was not collected and therefore could not be evaluated. This finding is partially supported by previous studies [22,23]. With the aim to identify predictors of *Streptococcus* domination, a prior study with 94 patients evaluated the following variables: age, sex, underlying diagnosis, prior antibiotic use, conditioning regimen intensity, T-cell depleted graft, stem cell source, and fever [23]. In this analysis, none of the evaluated variables reliably predicted Streptococcus domination [23]. However, in this same study, *Enterococcus* domination was increased three-fold in patients receiving metronidazole (HR 3.38; 95% CI 1.65–6.73; $p = 0.01$) and in patients with acute leukemia (HR 3.22; 95% CI 1.60–6.94; $p = 0.01$) [23]. Similar findings were reported by another study with 46 patients undergoing allo-HSCT [22]. Among seven potential predictors (age, sex, donor source, graft type, conditioning regimen intensity, and TBI-containing regimen), only acute leukemia was associated with *Enterococcus* domination (HR 2.48; 95% CI 1.13–5.45; $p = 0.024$) [22]. When combined with our findings, these data suggest that patient-level variables may not be reliable predictors of intestinal domination. Additionally, they suggest that predictors of intestinal domination may vary depending on the specific genus involved. Given the prognostic significance associated with some of these dominant genera, future studies evaluating other potential predictors are warranted.

The primary strength of this study is its comprehensive approach to evaluating the dynamics, predictors, and clinical implications of intestinal domination in Brazilian patients undergoing allo-HSCT. Nevertheless, our study presents some limitations. First, although we had pre-specified time points for stool sample collection, many patients were unable to provide samples at later stages, which significantly reduced the number of samples at D+90 and D+180. These relatively small sample sizes limit our ability to draw definitive conclusions regarding late post-transplantation microbial dynamics and to determine the prognostic significance of this unique domination fingerprint. Additionally, we did not collect stool samples over the engraftment period (defined as the first day of absolute neutrophil count $\geq 500/\mu L$ for 3 consecutive days, typically occurring between D+7 and D+21), which has been analyzed by prior studies [9,19–24]. This period is associated with peak microbiota dysbiosis and *Enterococcus* expansion. Therefore, the lack of samples over this period hinders an accurate comparison with other studies. Furthermore, due to data collection limitations in some centers, we were unable to evaluate: (1) the impact of intestinal domination in bloodstream infections, and (2) the role of antibiotics as a predictor of intestinal domination. Additionally, given the relatively small number of samples, analyses exploring clinical correlates of concurrent domination or stratified analyses were not performed. Also, all participating centers in our study were located within a single Brazilian state (São Paulo), which may limit the generalizability of our findings. Finally, our study does not provide mechanistic interpretations. Notwithstanding these limitations, our study is an important milestone in defining the dynamics and implications of intestinal domination events in Brazilian patients undergoing allo-HSCT, identifying a unique domination fingerprint that establishes important preliminary observations for future mechanistic investigations.

5. Conclusions

In a cohort of Brazilian patients undergoing allo-HSCT, we identified a unique intestinal domination fingerprint characterized by an extremely low prevalence of *Enterococcus* expansion. Although intestinal domination was a prevalent dysbiosis feature occurring in 78.2% of patients, our findings showed that intestinal domination by *Bacteroides*, *Akkermansia*, *Phascolarctobacterium,* and *Escherichia-Shigella* was not significantly associated with overall survival and aGvHD incidence. This pattern contrasts with international cohorts, in which *Enterococcus* often predominates and correlates with adverse outcomes, suggesting that the prognostic significance of intestinal domination during allo-HSCT may differ according to population-specific factors such as antibiotic practices and diet. These findings highlight the importance of considering geographic and environmental context when interpreting microbiota–outcome associations and support the need for future multi-center studies to elucidate the factors driving these distinct domination fingerprints.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jcm14238351/s1, Table S1: Proportion of Samples in Each Timepoint; Table S2: Details of Samples with Concurrent Domination by Two Distinct Genera; Table S3. Number of Intestinal Domination Events per Genus; Table S4: Multivariate Analyses Assessing Associations Between Intestinal Domination and Clinical Outcomes; and Table S5. Antibiotic Practices in Each Institution; Figure S1: Rarefaction curves showing the number of observed features (ASVs) as a function of sequencing depth for all stool samples included in the study; Figure S2. The Impact of intestinal domination on the cumulative incidence of severe aGvHD; and Figure S3: Sensitivity analysis to identify the optimal cutoff value for defining intestinal domination in relation to overall survival. Supplementary Methods—Predictors of Intestinal Domination [22,23,44].

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Data Availability Statement: The metadata from this study have been submitted to the NCBI Sequence Read Archive (BioProject PRJNA1357096).

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A Distinct Intestinal Domination Fingerprint in Patients Undergoing Allo-HSCT: Dynamics, Predictors and Implications on Clinical Outcomes

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Supplementary Table 1. Proportion of Samples in each Timepoint

Timepoint	N (%)
D-7	48 (25.0%)
D0	54 (28.1%)
D+30	28 (14.6%)
D+60	30 (15.6%)
D+90	20 (10.4%)
D+180	2 (1.0%)
aGvHD	10 (5.2%)

aGvHD = acute graft *versus* host disease

Supplementary Table 2. Details of Samples with Concurrent Domination by Two Distinct Genera

Time Point	Number of Sample	Codomination Genera
D-7	3	*Escherichia-Shigella + Veillonella*
		Akkermansia + Bacteroides
		Akkermansia + Bacteroides
D0	4	*Akkermansia + Bifidobacterium*
		Clostridia_vadinBB60_group + UCG-004
		Acidaminococcus + Parabacteroides
		Bacteroides + Escherichia-Shigella
D+30	3	*Bacteroides + Fusobacterium*
		Klebsiella + Uncultured

		Prevotella + Veillonella
D+60	4	*Bacteroides + Uncultured*
		Bacteroides + Phascolarctobacterium
		Escherichia-Shigella + Phascolarctobacterium
		Bacteroides + Parabacteroides
D+90	1	*Veillonella + Uncultured*
D+180	0	N/A
GvHD	2	*Fusobacterium + Phascolarctobacterium*
		Bacteroides + Parabacteroides
D = Day; GvHD = Graft *versus* host disease; N/A = Not Applicable.		

Supplementary Table 3. Number of Intestinal Domination Events per Genus

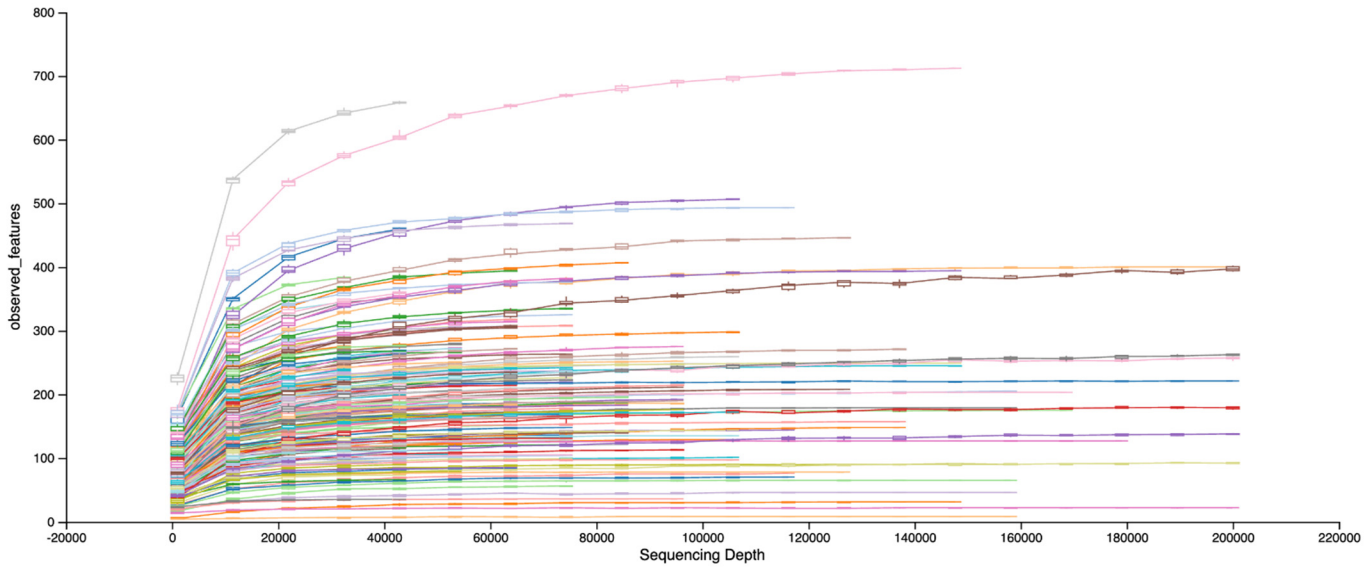
Genus	Number of Samples
Bacteroides	46
Akkermansia	19
Phascolarctobacterium	16
Escherichia-Shigella	14
Prevotella	8
Parabacteroides	7
Veillonella	5
Acidaminococcus	4
Campylobacter	3
Fusobacterium	3
Klebsiella	3
Uncultured	3
Alistipes	2
Bilophila	2
Clostridia_vadinBB60_group	2
Dialister	2
UBA1819	1
Bifidobacterium	1
Enterococcus	1
Faecalibacterium	1
Haemophilus	1
Lactobacillus	1
Staphylococcus	1
UCG-004	1

Supplementary Table 4. Multivariate Analyses Assessing Associations Between Intestinal Domination and Clinical Outcomes

Genera	Overall Survival* HR [95% CI]; P value	aGvHD Cumulative Incidence* HR [95% CI]; P value

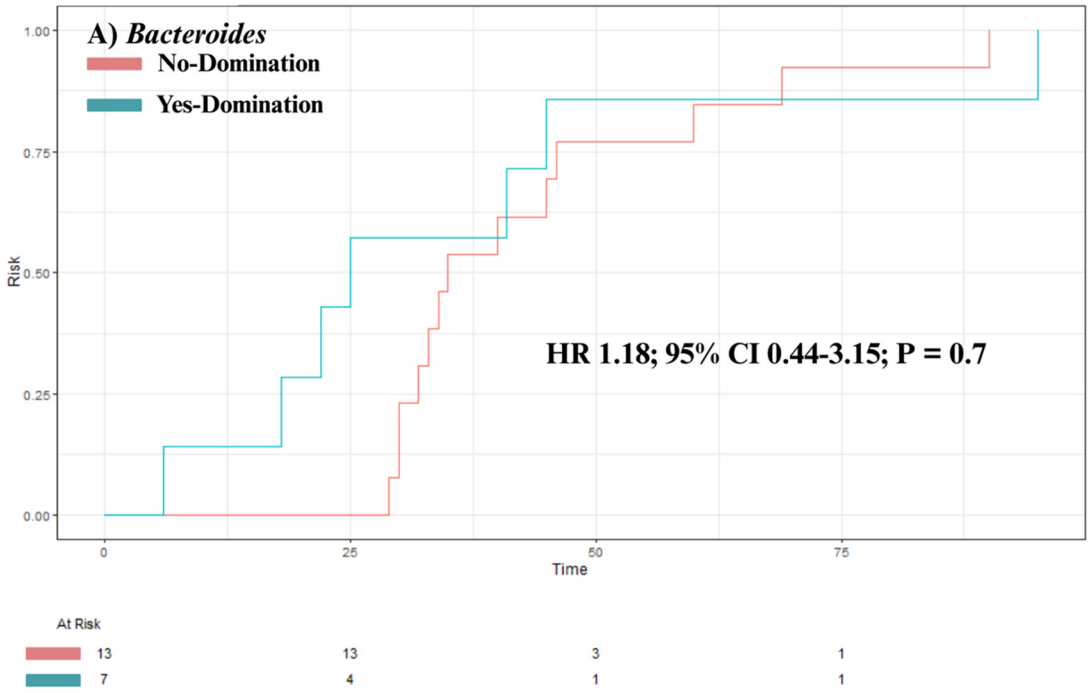
Bacteroides	1.56 [0.42-5.85]; P = 0.5	0.64 [0.29-1.40]; P = 0.3
Akkermansia	1.27 [0.16-10.2]; P = 0.8	0.41 [0.14-1.26]; P = 0.12
Phascolarctobacterium	1.66 [0.15-18.3]; P = 0.7	1.66 [0.58-4.76]; P = 0.3
Escherichia-Shigella	0.52 [0.07-3.91]; P = 0.5	1.68 [0.68-4.14]; P = 0.3
aGvHD = acute graft-versus-host-disease; CI = Confidence Interval; HR = Hazard Ratio; * = multivariate analyses adjusted for the following potential cofounders – age, sex, center, underlying diagnosis, conditioning regimen, donor sex, stem cell source, and donor type		

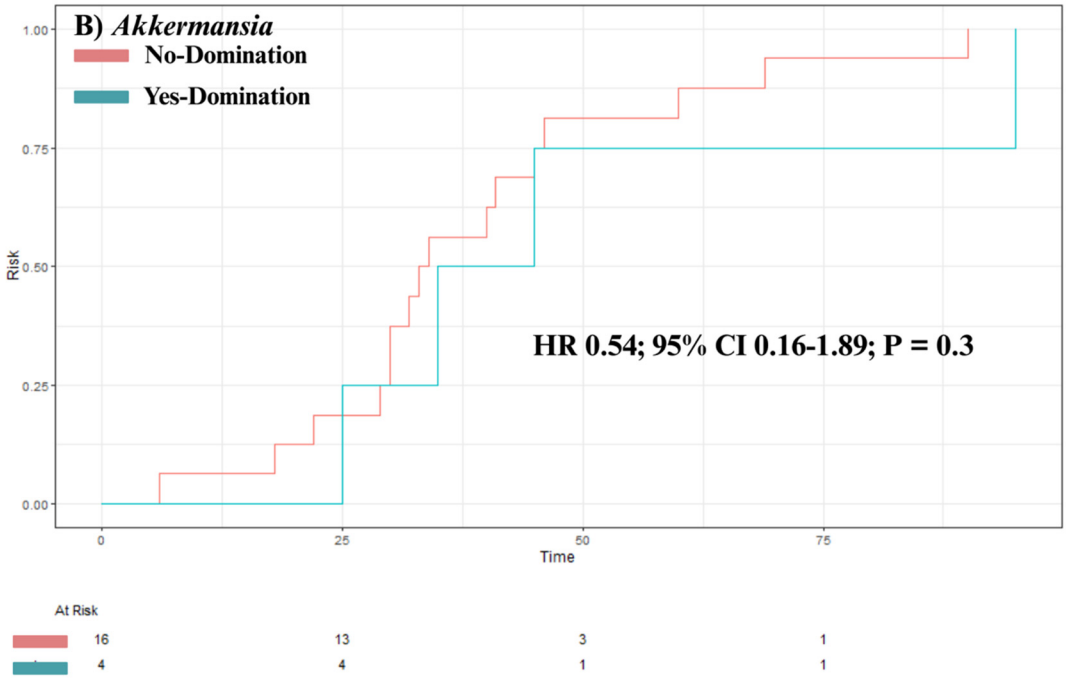
Supplementary Figure 1.

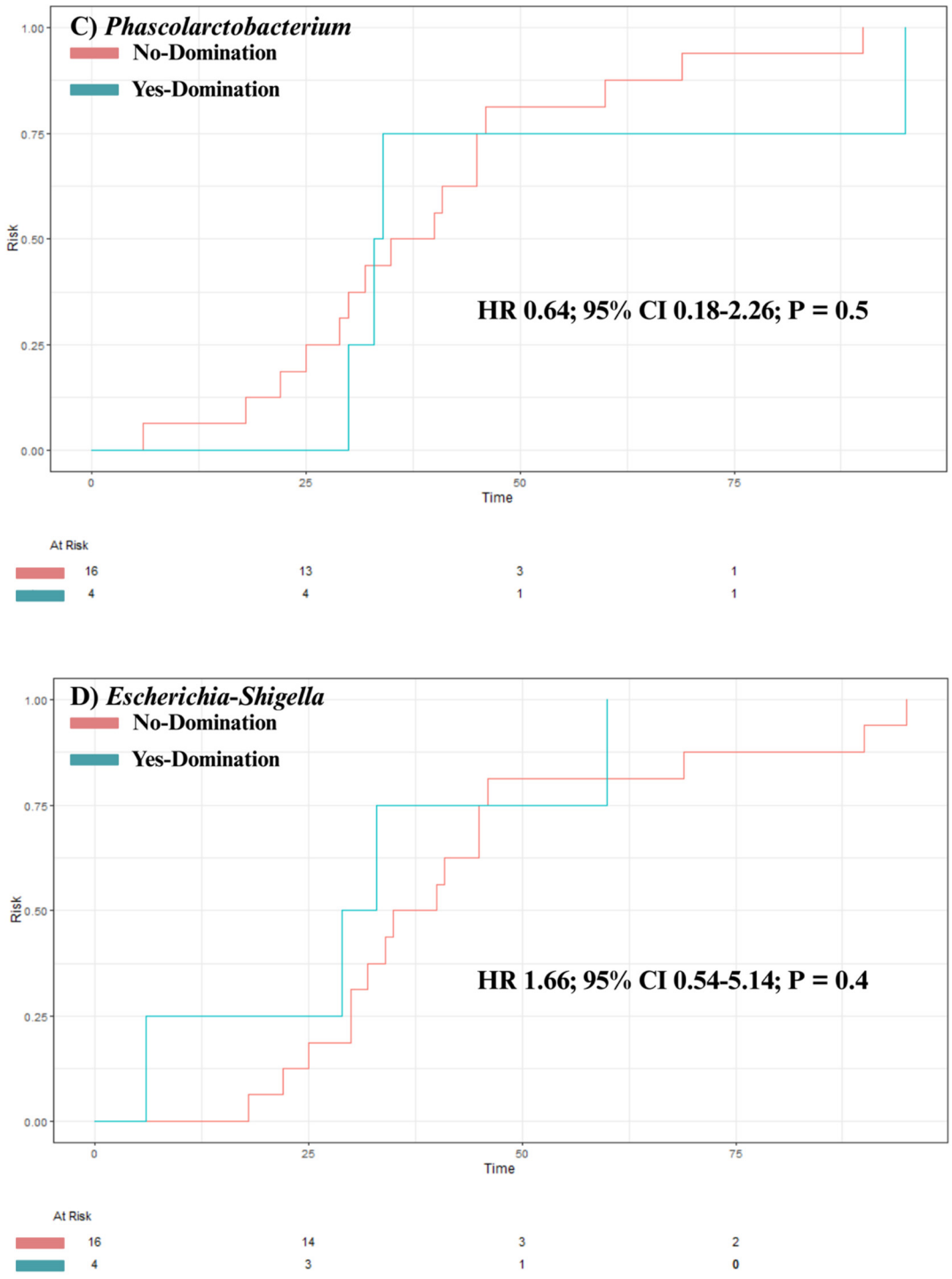


Supplementary Figure 1. Rarefaction curves showing the number of observed features (ASVs) as a function of sequencing depth for all stool samples included in the study. Each line represents an individual sample. ASVs = amplicon sequence variants.

Supplementary Figure 2

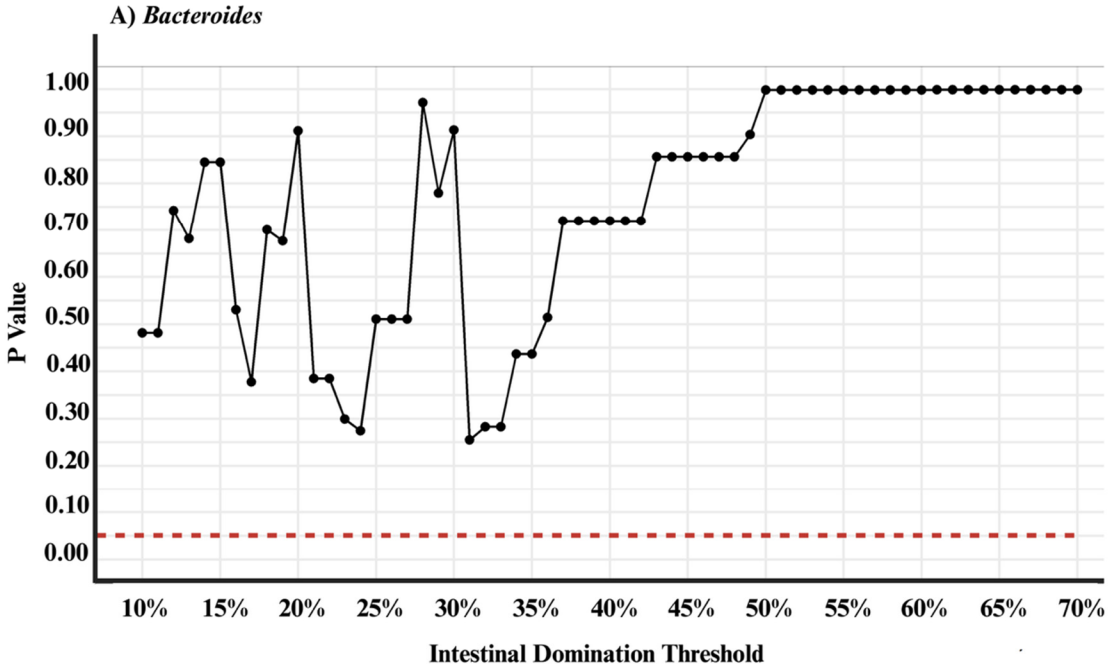

A) Bacteroides
No-Domination
Yes-Domination
HR 1.18; 95% CI 0.44-3.15; P = 0.7
Risk
Time
1.00
0.75
0.50
0.25
0.00
0
25
50
75
At Risk
13
13
3
1
7
4
1
1


B) Akkermansia
No-Domination
Yes-Domination
HR 0.54; 95% CI 0.16-1.89; P = 0.3
Risk
Time
1.00
0.75
0.50
0.25
0.00
0
25
50
75
At Risk
16
13
3
1
4
4
1
1

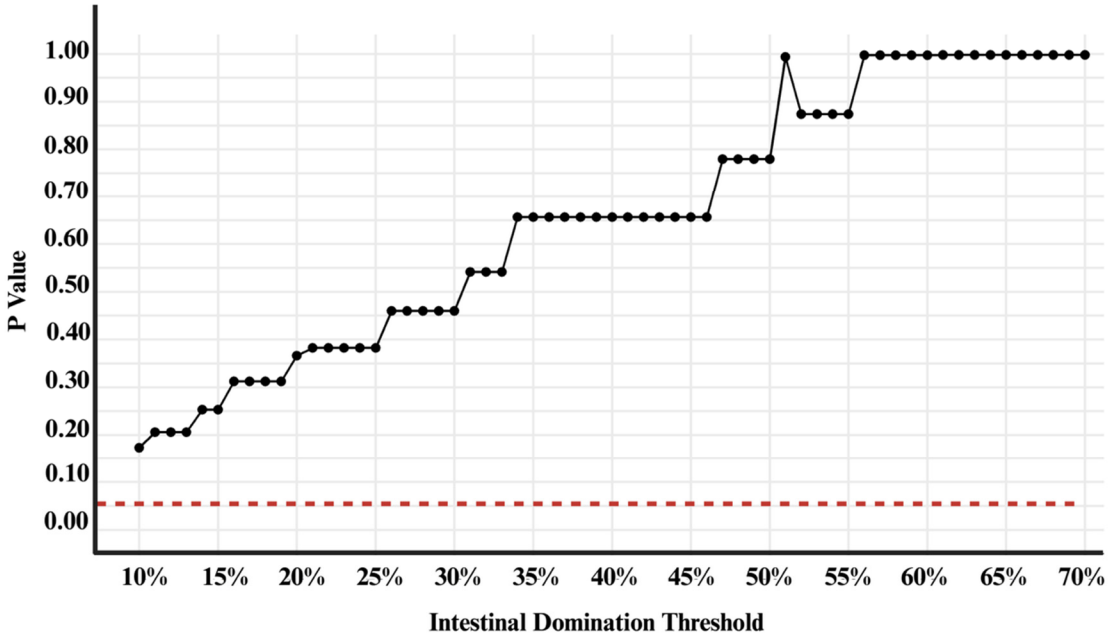


Supplementary Figure 2. The impact of intestinal domination on the cumulative incidence of severe aGvHD. **A)** *Bacteroides*. **B)** *Akkermansia*. **C)** *Phascolarctobacterium*. **D)** *Escherichia-Shigella*. HR = Hazard Ratio. CI = Confidence interval.

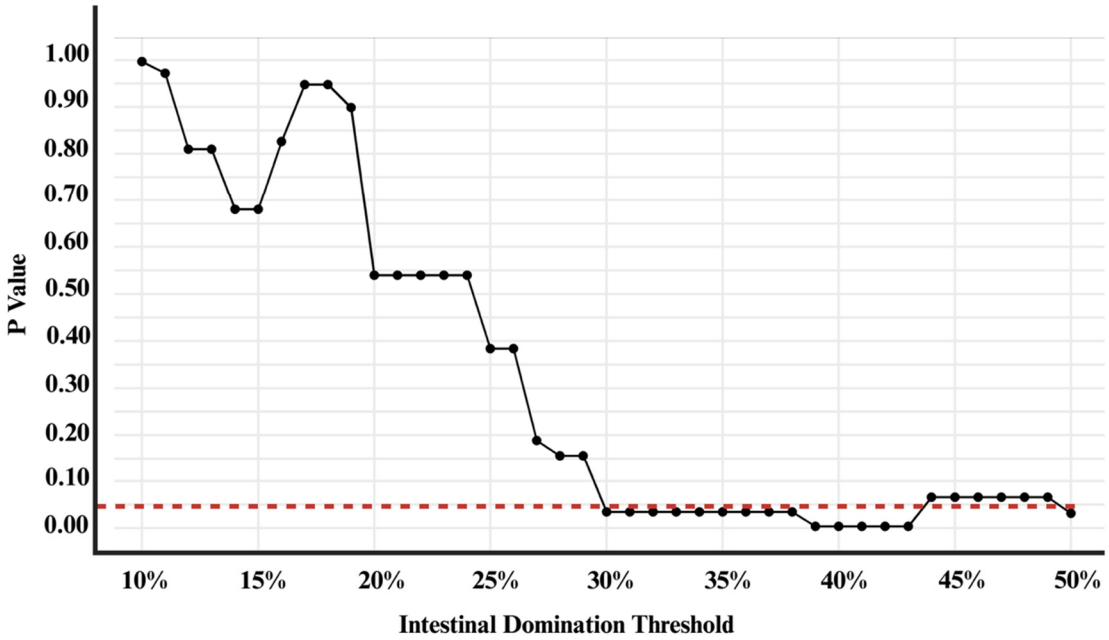
Supplementary Figure 3

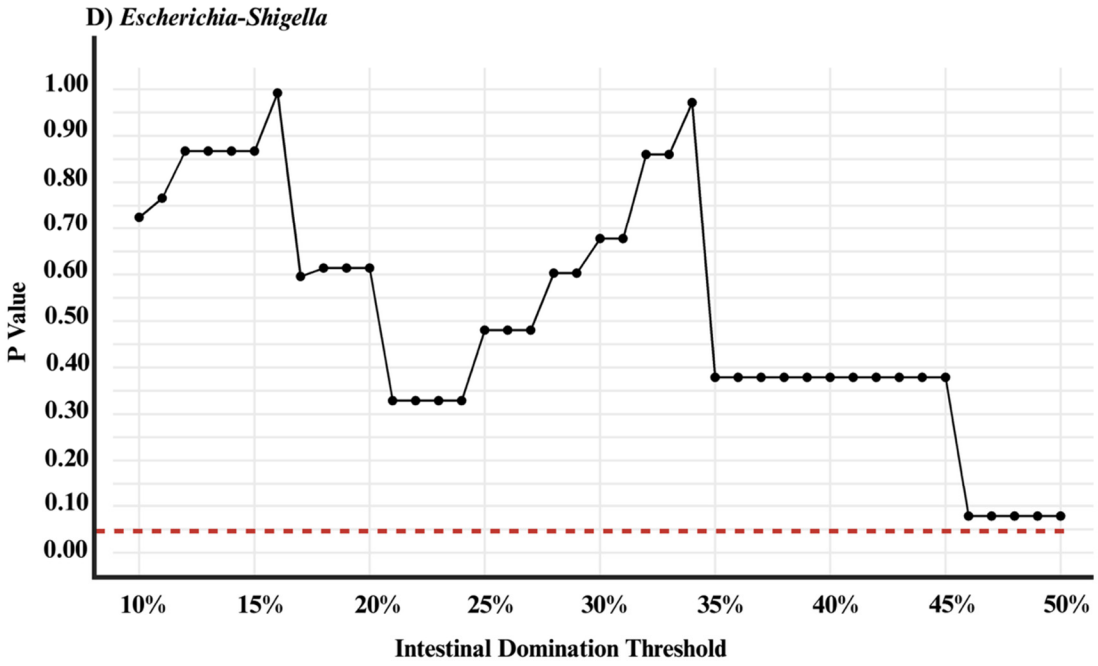


B) *Akkermansia*



C) *Phascolarctobacterium*





Supplementary Figure 3. Sensitivity analysis to identify the optimal cutoff value for defining intestinal domination in relation to overall survival. (A) *Bacteroides*. (B) *Akkermansia*. (C) *Phascolarctobacterium*. (D) *Escherichia-Shigella*. The y-axis represents the p-value of the Cox regression model for each cutoff tested. The red line represents the significance threshold (P = 0.05).

Supplementary Methods – Predictors of Intestinal Domination

Patients were stratified according to the presence or absence of intestinal domination by any genera or by the four most common genera in any time point. Potential predictors of intestinal domination were selected based on prior studies and included: 1) age, 2) sex, 3) conditioning regimen, 4) stem cell source, 5) TBI (total body irradiation)-containing regimen; and 6) underlying diagnosis (22,23). Similar to prior studies, the underlying diagnosis was classified as acute leukemia vs. others (22,23). The conditioning regimens were classified as 1) myeloablative, 2) nonmyeloablative, and 3) reduced-intensity conditioning per standardized definitions (24). Due to data collection limitations, this study was not able to consider

antibiotics and prior infections as potential predictors of intestinal domination (22,23). However, antibiotic practices in each center are shown in **Supplementary Table 5**.

Supplementary Table 5. Antibiotic Practices in Each Institution

Center	Antibiotic Prophylaxis	First-line Neutropenic Fever	Alternative in Penicillin-allergic Patients
HB-FUNFARME	Levofloxacin	Piperacillin-Tazobactam	Meropenem
HAC	Levofloxacin (Adults) None (Children)	Piperacillin-Tazobactam	Cefepime
HCB	None	Cefepime	Meropenem
BP	None	3rd Generation Cephalosporins	Meropenem

BP = Hospital Beneficencia Portuguesa de Sao Paulo, HB-FUNFARME = Hospital de Base of Fundacao Faculdade Regional de Medicina; HAC = Hospital Amaral Carvalho; HCB = Hospital de Cancer de Barretos.

5.3.2. Manuscrito II – Artigo Original – Avaliação de Zonulina no Alo-TM

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Unraveling the Role of Zonulin in Allogeneic Hematopoietic Stem Cell Transplantation: A Multicenter Study

Alexandre Soares Ferreira Junior [1,†], Nathalia Linares Silva [1,†], Danielle Amanda Niz Alvarez [1], Larissa da Silva Souza [1], Luiza Dias Machado [1], Bianca Fernanda Rodrigues da Silva [1], Welinton Yoshio Hirai [2], Rozana Mesquita Ciconelli [3], Joao Victor Piccolo Feliciano [4], Iago Colturato [5], George Maurício Navarro Barros [6], Phillip Scheinberg [3] and Gislane Lelis Vilela de Oliveira [1,*]

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Abstract

The role of zonulin as a biomarker of intestinal permeability in the allogeneic hematopoietic stem cell transplantation (allo-HSCT) setting remains poorly understood. In this study, we aimed to evaluate serum zonulin dynamics, identify its predictors, and assess its prognostic significance in patients undergoing allo-HSCT. This multicenter, prospective cohort study was conducted across four Brazilian hospitals. Eligible participants were patients aged ≥12 years who provided at least one blood sample during the allo-HSCT course. A control group of 15 healthy adult individuals was also included. Serum zonulin levels were quantified using enzyme-linked immunosorbent assay multiple times over the allo-HSCT course. Outcomes included acute graft-versus-host disease, overall survival, and bloodstream infections. A total of 477 blood samples were collected from 140 patients. Compared with the control group, zonulin levels were persistently elevated at all evaluated time points throughout the allo-HSCT course. However, no significant differences were observed among the different time points assessed during transplantation. No clinical or transplantation-related characteristics were identified as significant predictors of elevated zonulin levels. Finally, zonulin did not demonstrate prognostic value for allo-HSCT-related outcomes. Future studies should investigate whether other intestinal permeability biomarkers have prognostic relevance in the allo-HSCT setting.

Keywords: intestinal barrier function; stem cell transplantation; gastrointestinal microbiome; intestinal mucosa; graft-versus-host-disease; zonulin

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1. Introduction

In patients undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT), adverse clinical outcomes, including graft-versus-host disease (GvHD) and oral mucositis, have been associated with damage to the gastrointestinal barrier [1–3]. The gastrointestinal barrier is a complex and dynamic structure organized into three main layers: (1) the mucus layer; (2) the intestinal epithelium; and (3) the lamina propria. These layers act synergistically to control intestinal permeability through both transcellular and paracellular pathways, thereby maintaining selective permeability (regulated transport of microorganisms, metabolites, and antigens) [4–6]. The paracellular pathway, which involves transport through the intercellular spaces between adjacent epithelial cells, has been classically implicated in the development of several disease states [5,7]. This route is primarily regulated by tight junctions, which are multiprotein complexes. Among the factors involved in the regulation of the paracellular route, a key modulator of tight junction integrity that promotes increased paracellular permeability is zonulin [8].

Zonulin, also known as pre-haptoglobin 2, is a multifunctional protein produced by intestinal epithelial cells that acts similarly to the Zot enterotoxin produced by *Vibrio cholerae* [7]. Zonulin increases paracellular permeability by activating epidermal growth factor (EGF) receptors in enterocytes and promoting tight junction disassembly [7]. Zonulin has been increasingly implicated in a broad spectrum of immune-mediated, metabolic, and inflammatory conditions [7]. By modulating the paracellular pathway, zonulin regulates the trafficking of multiple potential antigens and influences the balance between immune tolerance and alloreactivity to non-self antigens [7]. For instance, in patients with celiac disease, accumulating evidence has identified zonulin as an important factor in disease pathogenesis [7]. In genetically susceptible individuals, zonulin expression is upregulated in response to gluten ingestion, leading to increased paracellular permeability and enhanced exposure of the immune system to luminal antigens [7]. Zonulin has also been investigated as a potential diagnostic and prognostic biomarker. In patients with cirrhosis, zonulin levels have been shown to predict the severity of hepatic decompensation, as well as the risk of acute kidney injury and hepatorenal syndrome [9]. Other diseases that have been associated with zonulin levels include type 1 diabetes, obesity, metabolic syndrome, inflammatory bowel diseases, and cancer [7,10].

Although zonulin has been extensively evaluated in other clinical settings, its significance in patients with hematologic malignancies and those undergoing HSCT (auto and allo-HSCT) remain largely unknown. Prior studies have primarily focused on pediatric allo-HSCT patients and animal models [11,12]. The evaluation of zonulin in allo-HSCT is particularly relevant, as these patients frequently experience substantial gastrointestinal barrier damage due to multiple transplantation-related factors, including conditioning regimens, the use of total body irradiation, and intestinal dysbiosis [2,13–16]. Moreover, allo-HSCT continues to be associated with considerable morbidity and mortality. Finally, zonulin could be readily translated into clinical practice, as it represents an accessible and easily measurable surrogate marker of intestinal permeability [7,17]. Thus, the assessment of zonulin may contribute to risk stratification, outcome prediction, and the identification of potential therapeutic targets. In this multicenter Brazilian study, we aimed to evaluate the dynamics, determinants, and prognostic significance of zonulin in patients undergoing allo-HSCT. As demonstrated in the following sections, although we identified persistently elevated zonulin levels, zonulin did not emerge as a significant predictor of allo-HSCT–related outcomes.

2. Results

2.1. Demographic and Clinical Characteristics

The number of patients meeting the inclusion criteria (eligible patients included individuals undergoing any type of allo-HSCT except umbilical cord blood transplantation) was 140. These patients had 477 blood samples collected throughout predetermined allo-HSCT time points (D−7 [7 days prior to allo-HSCT], D0, D+30, D+60, D+90, D+180, and at the time of GvHD suspicion). Overall, most samples (70.2%; n = 335) were collected during the early time points (D−7 to D+30; see Supplementary Table S1 for details on the number of samples per time point and center). The demographics and clinical characteristics are shown in Table 1. Overall, the median age was 41 years (range, 12–73), and most patients were male (n = 79, 56.4%). The most common underlying diagnosis was leukemia (n = 100, 73.5%). In our overall cohort, mean follow-up time was 11 months (SD [Standard Deviation] ± 9 months).

Table 1. Demographic and Clinical Characteristics.

Variables	HB-FUNFARME (N = 40)	HAC (N = 48)	HCB (N = 10)	BP (N = 42)	Total (140)
Age (years)					
Median (Range)	39 (18–66)	43 (13–68)	43 (19–61)	43 (12–73)	41 (12–73)
Mean (SD)	41 (14)	41 (15)	41 (15)	46 (18)	42 (16)
Biological Sex					
Female	14 (35.0%)	22 (45.8%)	6 (60.0%)	19 (45.2%)	61 (43.6%)
Male	26 (65.0%)	26 (54.2%)	4 (40.0%)	23 (54.8%)	79 (56.4%)
Ethnicity					
Afro-descendants	9 (22.5%)	25 (52.1%)	4 (40.0%)	14 (33.3%)	52 (37.1%)
Asian	1 (2.5%)	0 (0.0%)	0 (0.0%)	1 (2.4%)	2 (1.4%)
Caucasian	30 (75.0%)	23 (47.9%)	6 (60.0%)	27 (64.3%)	86 (61.4%)
Weight (kg)					
Median (Range)	79 (51–115)	73 (47–109)	68 (43–81)	73 (46–130)	73 (43–130)
Mean (SD)	78 (15)	76 (15)	66 (13)	74 (19)	75 (16)
BMI					
Median (Range)	26.1 (15.8–40.7)	26.5 (17.5–37.6)	22.8 (17.5–38.0)	25.2 (17.0–38.4)	25.8 (15.8–40.7)
Mean (SD)	26.6 (4.9)	27.1 (4.6)	24.7 (6.2)	26.3 (5.1)	26.5 (5.0)
Height (cm)					
Median (Range)	173 (151–191)	167 (144–195)	166 (146–188)	166 (150–189)	168 (144–195)
Mean (SD)	171 (10)	167 (10)	165 (12)	168 (10)	168 (10)
Underlying Diagnosis					
Leukemias	30 (75.0%)	36 (75.0%)	6 (60.0%)	31 (73.8%)	103 (73.6%)
Lymphomas	2 (5.0%)	1 (2.1%)	3 (30.0%)	1 (2.4%)	7 (5.0%)
Others	8 (20.0%)	11 (22.9%)	1 (10.0%)	10 (23.8%)	30 (21.4%)
TBI					
No-TBI	29 (72.5%)	21 (43.8%)	2 (20.0%)	18 (42.9%)	70 (50.0%)
Yes-TBI	11 (27.5%)	27 (56.3%)	8 (80.0%)	24 (57.1%)	70 (50.0%)

Table 1. *Cont.*

Variables	HB-FUNFARME (N = 40)	HAC (N = 48)	HCB (N = 10)	BP (N = 42)	Total (140)
Conditioning Regimen					
Myeloablative	14 (35.0%)	23 (47.9%)	2 (20.0%)	12 (28.6%)	51 (36.4%)
Non Myeloablative	5 (12.5%)	2 (4.2%)	0 (0.0%)	14 (35.9%)	21 (15.0%)
Reduced Intensity	21 (52.5%)	23 (47.9%)	8 (80.0%)	16 (38.1%)	68 (48.6%)
HLA Match Status					
MRD	18 (45.0%)	13 (27.1%)	1 (10.0%)	10 (23.8%)	42 (30.0%)
MUD	1 (2.5%)	9 (18.8%)	1 (10.0%)	6 (14.3%)	17 (12.1%)
Haploidentical	21 (52.5%)	23 (47.9%)	6 (60.0%)	21 (50.0%)	71 (50.7%)
MMUD	0 (0.0%)	3 (6.3%)	0 (0.0%)	2 (4.8%)	5 (3.6%)
Mismatched Related Donor (5–10 HLA match)	0 (0.0%)	0 (0.0%)	2 (20.0%)	3 (7.1%)	5 (3.6%)
Donor Sex					
Male	24 (60.0%)	34 (70.8%)	7 (70.0%)	25 (59.5%)	90 (64.3%)
Female	16 (40.0%)	14 (29.2%)	3 (30.0%)	17 (40.5%)	50 (35.7%)
Stem Cell Source					
Peripheral Blood	37 (92.5%)	28 (58.3%)	9 (90.0%)	36 (85.7%)	110 (78.6%)
Bone Marrow	3 (7.5%)	20 (41.7%)	1 (10.0%)	5 (11.9%)	29 (20.7%)
Others	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (2.4%)	1 (0.7%)
Prior Stem Cell Transplant					
No	39 (97.5%)	45 (93.8%)	6 (60.0%)	40 (95.2%)	130 (92.9%)
Yes	1 (2.5%)	3 (6.3%)	4 (40.0%)	2 (4.8%)	10 (7.1%)

BMI = Body Mass Index; HLA = Human Leukocyte Antigens; MMUD = Mismatched Unrelated Donor; MRD = Matched Related Donor; MUD = Matched Unrelated Donor; N = Number; SD = Standard Deviation; TBI = Total Body Irradiation; HB-FUNFARME = Hospital de Base of the Fundação Faculdade Regional de Medicina; HAC = Hospital Amaral Carvalho; HCB = Hospital de Câncer de Barretos; BP = Hospital Beneficência Portuguesa de São Paulo.

2.2. Zonulin Dynamics During Allo-HSCT

Zonulin dynamics during allo-HSCT is shown in Figure 1. We also included a control group of 15 healthy adults, defined as individuals without chronic inflammatory diseases or recent use of antibiotics, anti-inflammatory drugs, or immunosuppressants, to better characterize zonulin dynamics. Overall, patients showed no significant difference in zonulin levels over the allo-HSCT course. However, when compared with the healthy control group (21 ng/mL), zonulin levels were persistently elevated at all allo-HSCT time points: D−7 (64 ng/mL; $p < 0.0001$), D0 (58 ng/mL, $p < 0.0001$), D+30 (54 ng/mL; $p = 0.0003$), D+60 (57 ng/mL; $p < 0.0001$), D+90 (61 ng/mL; $p < 0.0001$), D+180 (60 ng/mL; $p = 0.0024$), and GvHD (55 ng/mL; $p = 0.0001$). We also assessed zonulin dynamics over the allo-HSCT course stratified by the presence of GvHD and GI (Gastrointestinal)-GvHD (see Supplementary Figure S1A,B). In both analyses, no significant differences in zonulin trajectories were identified.

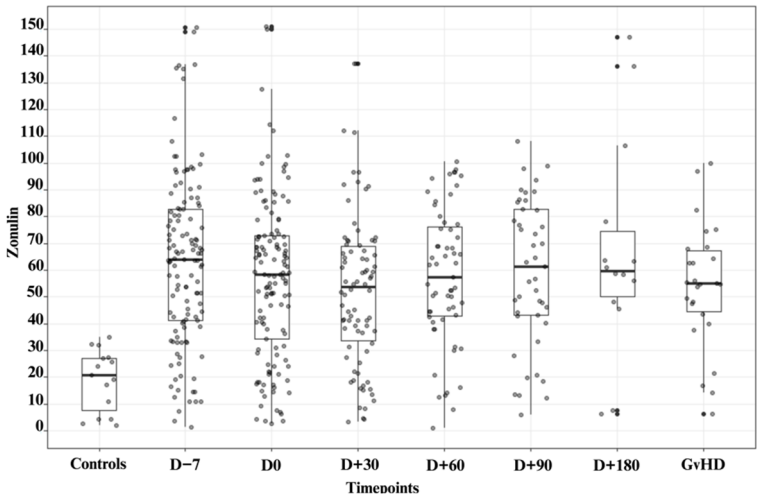


Figure 1. Temporal dynamics of zonulin during the allo-HSCT course (analyzed using paired Wilcoxon tests). D = day; GvHD = graft-versus-host disease.

2.3. Predictors of Increased Zonulin Levels

To identify predictors of increased zonulin levels, patients were stratified at each time point into high- and low-zonulin groups based on the median zonulin value at the corresponding time point. Only early time points (up to D+30) were included in this analysis, given our interest in evaluating early zonulin levels as a potential prognostic biomarker. Among the variables analyzed as potential predictors of increased zonulin levels, at D−7, D0 and D+30, none were identified as significant (see Table 2). Additionally, none of the variables were significant predictors when analyzing Δ(delta)Zonulin (the difference between D0 and D−7).

Table 2. Predictors of Increased Zonulin Levels at Different Time Points in the Overall Cohort of Allo-HSCT Patients.

	D−7		D0		D+30		ΔP	
Variables	**OR (95% CI) ***	***p* Value**	**OR (95% CI) ***	***p* Value**	**OR (95% CI) ***	***p* Value**	**OR (95% CI) ***	***p* Value**
Hospital								
HAC	–		–		–		–	
BP	0.64 (0.27, 1.51)	0.3	0.61 (0.26, 1.40)	0.2	0.66 (0.20, 2.10)	0.5	2.11 (0.88, 5.14)	0.10
HB-FUNFARME	1.67 (0.65, 4.46)	0.3	1.06 (0.42, 2.69)	>0.9	1.56 (0.50, 4.96)	0.4	1.57 (0.58, 4.26)	0.4
HCB	0.22 (0.03, 0.99)	0.072	0.35 (0.07, 1.41)	0.2	2.69 (0.49, 21.2)	0.3	0.43 (0.06, 1.95)	0.3
Weight (Kg)	0.99 (0.97, 1.01)	0.4	1.01 (0.99, 1.04)	0.2	1.01 (0.99, 1.04)	0.3	1.02 (1.00, 1.04)	0.11
Height (Cm)	0.97 (0.94, 1.01)	0.14	0.99 (0.96, 1.02)	0.5	0.98 (0.98, 1.02)	0.4	1.01 (0.98, 1.05)	0.6
Biological Sex								
Female	–		–		–		–	
Male	0.59 (0.29, 1.21)	0.2	0.59 (0.29, 1.18)	0.14	0.44 (0.17, 1.10)	0.082	0.82 (0.40, 1.71)	0.6
Prior Stem Cell Transplant								
No	–		–		–		–	
Yes	1.00 (0.26, 3.78)	>0.9	1.58 (0.43, 6.44)	0.5	3.27 (0.70, 23.4)	0.2	0.85 (0.21, 3.13)	0.8
TBI								
No-TBI	–		–		–		–	
Yes-TBI	0.68 (0.33, 1.37)	0.3	0.63 (0.31, 1.25)	0.2	0.85 (0.34, 2.09)	0.7	0.69 (0.33, 1.44)	0.3

Table 2. *Cont.*

	D−7		D0		D+30		ΔP	
Variables	**OR (95% CI) ***	***p* Value**	**OR (95% CI) ***	***p* Value**	**OR (95% CI) ***	***p* Value**	**OR (95% CI) ***	***p* Value**
Conditioning Regimen								
Myeloablative	–		–		–		–	
Reduced Intensity	0.94 (0.43, 2.05)	0.9	0.64 (0.29, 1.36)	0.2	1.00 (0.38, 2.65)	>0.9	0.95 (0.42, 2.06)	0.9
Non Myeloablative	0.66 (0.22, 1.95)	0.5	0.69 (0.23, 2.03)	0.5	1.20 (0.30, 5.00)	0.9	1.12 (0.36, 3.49)	0.8
Age at Allo-HSCT	1.00 (0.98, 1.02)	>0.9	1.00 (0.97, 1.02)	0.7	1.00 (1.00, 1.00)	0.8	1.00 (0.98, 1.02)	>0.9
Underlying Diagnosis								
Leukemia	–		–		–			
Others	0.79 (0.36, 1.72)	0.5	1.11 (0.50, 2.46)	0.8	1.27 (0.46, 3.59)	0.7	0.59 (0.25, 1.34)	0.2
Prior Antibiotics Use	N/A	N/A	0.47 (0.14, 1.40)	0.2	0.70 (0.28, 1.70)	0.4	N/A	N/A
Antibiotics Use for ≥3D Prior to Sample Collection	N/A	N/A	1.25 (0.64, 2.55)	0.5	0.70 (0.28, 1.70)	0.4	N/A	N/A
Beta-Lactams	N/A	N/A	0.80 (0.19, 3.16)	0.7	0.63 (0.25, 1.54)	0.3	N/A	N/A
Fluoroquinolones	N/A	N/A	1.02 (0.04, 26.1)	>0.9	2.06 (0.38, 15.5)	0.4	N/A	N/A
Glycopeptides	N/A	N/A	0.50 (0.02, 5.35)	0.6	1.33 (0.44, 4.15)	0.6	N/A	N/A
Anti-anaerobics	N/A	N/A	0.80 (0.19, 3.16)	0.7	0.63 (0.25, 1.54)	0.3	N/A	N/A

Allo-HSCT = Allogeneic hematopoietic stem cell transplantation; CI = Confidence interval; D = day; N/A = Not applicable; OR = Odds ratio; TBI = Total Body Irradiation; ΔP = Difference between permeability in periods D−7 and D0; * = Logistic regression analysis was performed to identify predictors of increased zonulin levels.

2.4. Associations Between Zonulin and Clinical Outcomes

Among the 140 patients included in this study, 31 (23.0%) died during the follow-up period, 60 (45.0%) were diagnosed with GvHD, including 24 (17.0%) with GI GvHD, and 54 (38.6%) had BSI (bloodstream infection). Associations between each outcome and zonulin levels were evaluated by analyzing zonulin both as a continuous variable and as a categorical variable (low vs. high). For the categorical analyses, patients were stratified according to the median zonulin value at each time point. In addition, when zonulin was analyzed as a categorical variable, sensitivity, specificity, and predictive values were calculated for each outcome (see Supplementary Table S2).

2.4.1. Associations Between Zonulin and GvHD

We first categorized patients based on the median zonulin level into high vs. low zonulin groups. No significant associations were identified with the cumulative incidence of GvHD (D−7: HR [Hazard Ratio] 1.13; 95% CI [Confidence Interval] 0.67–1.91; $p = 0.6$; D0: HR 0.96; 95% CI 0.58–1.59; $p = 0.9$; D+30: HR 0.95; 95% CI 0.47–1.91; $p > 0.9$; ΔZonulin: HR 0.95; 95% CI 0.56–1.60; $p = 0.8$; see Figure 2A–D), and severe GvHD (D−7: HR 0.74; 95% CI 0.37–1.47; $p = 0.4$; D0: HR 0.88; 95% CI 0.45–1.71; $p = 0.7$; D+30: HR 0.75; 95% CI 0.28–1.00; $p = 0.6$; ΔZonulin: HR 1.54; 95% CI 0.76–3.14; $p = 0.2$ see Figure 3A–D). Similarly, no significant associations with the cumulative incidence of GvHD or severe GvHD were found when zonulin levels were analyzed as a continuous variable (see Table 3).

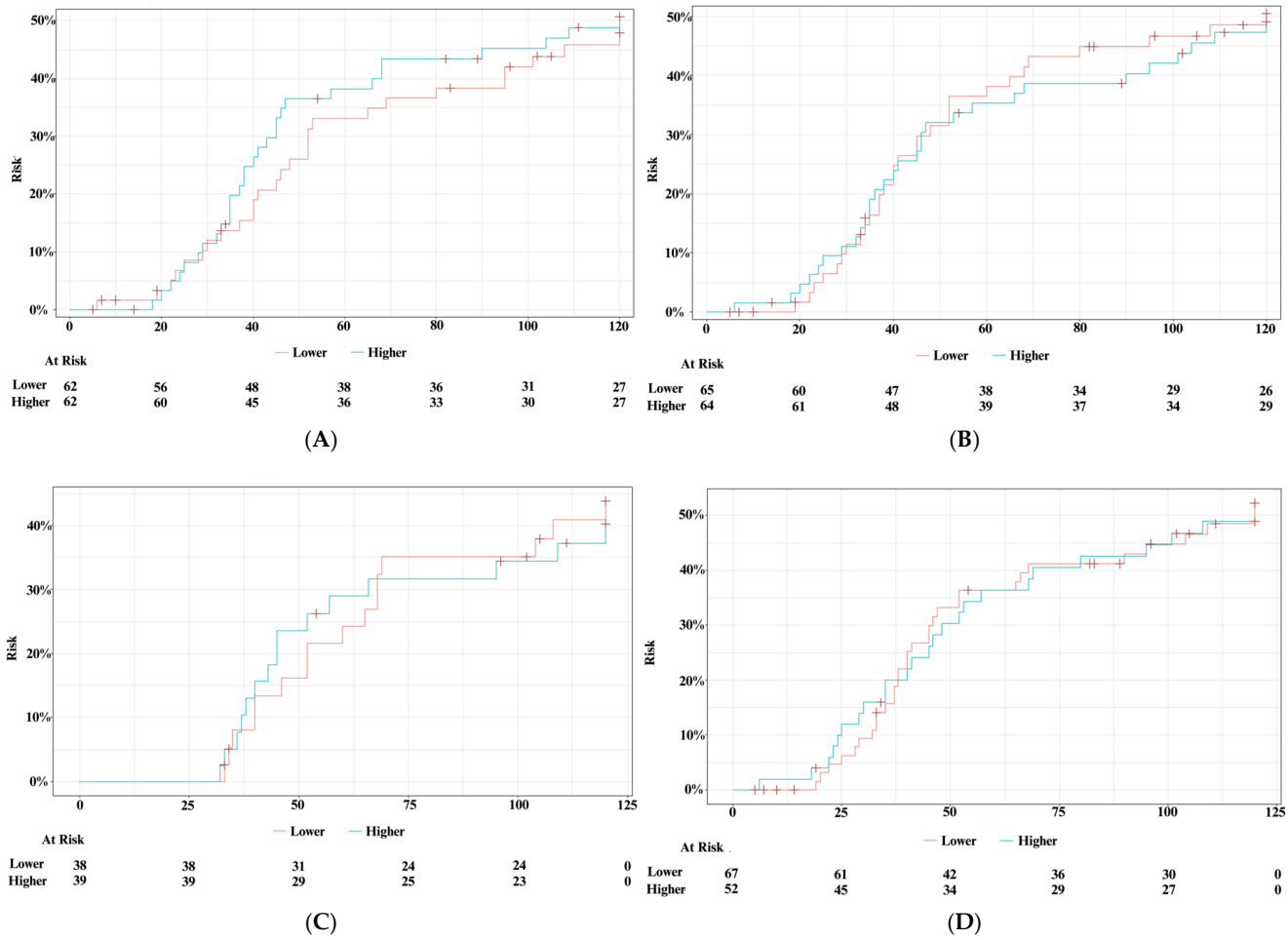


Figure 2. (**A**) Association between zonulin levels (low [red] vs. high [blue]) and the cumulative incidence of GvHD at D−7 (analyzed using Cox proportional hazards regression). D = day; GvHD = graft-versus-host disease. (**B**) Association between zonulin levels (low [red] vs. high [blue]) and the cumulative incidence of GvHD at D0 (analyzed using Cox proportional hazards regression). D = day; GvHD = graft-versus-host disease. (**C**) Association between zonulin levels (low [red] vs. high [blue]) and the cumulative incidence of GvHD at D+30 (analyzed using Cox proportional hazards regression). D = day; GvHD = graft-versus-host disease. (**D**). Association between zonulin levels (low [red] vs. high [blue]) and the cumulative incidence of GvHD using ΔZonulin (D0 minus D−7. D = day; analyzed using Cox proportional hazards regression). D = day; GvHD = graft-versus-host disease.

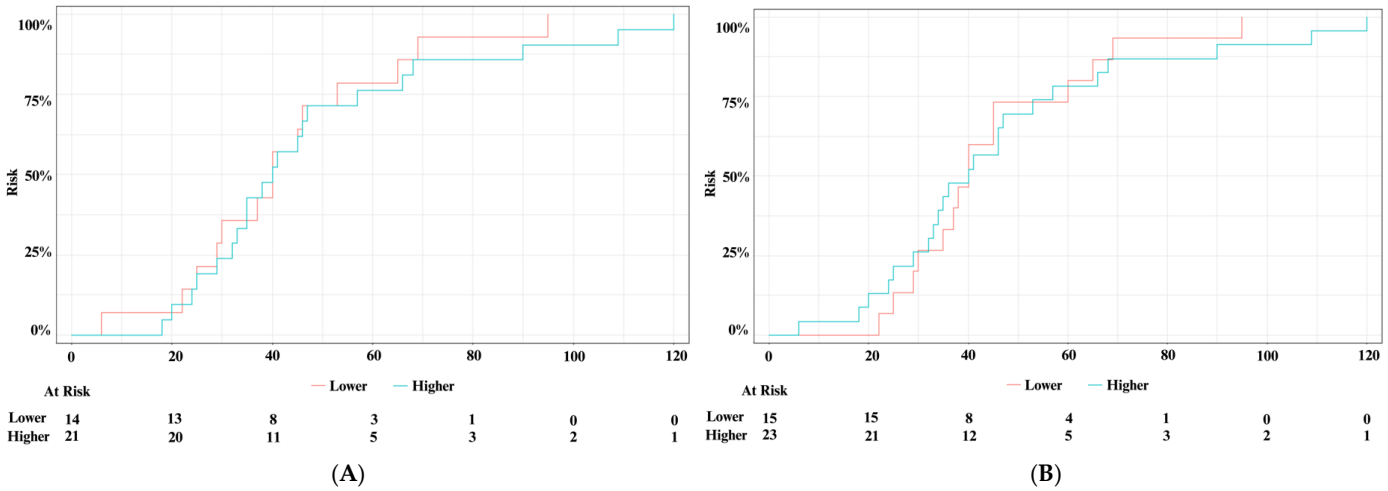
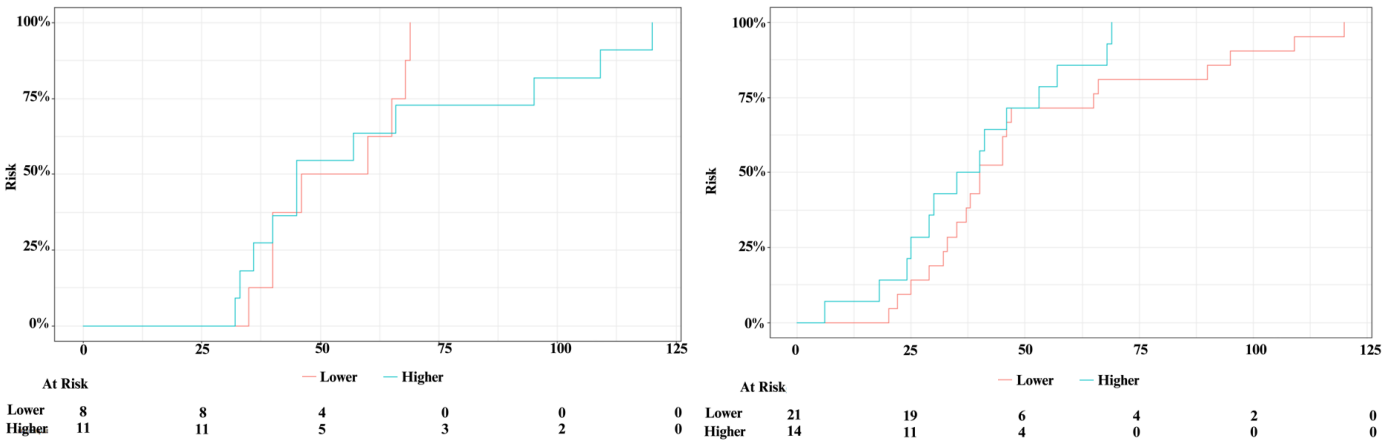


Figure 3. *Cont.*



(**C**) (**D**)

Figure 3. (**A**). Association between zonulin levels (low [red] vs. high [blue]) and the cumulative incidence of severe GvHD at D−7 (analyzed using Cox proportional hazards regression). D = day; GvHD = graft-versus-host disease. (**B**) Association between zonulin levels (low [red] vs. high [blue]) and the cumulative incidence of severe GvHD at D0 (analyzed using Cox proportional hazards regression). D = day; GvHD = graft-versus-host disease. (**C**) Association between zonulin levels (low [red] vs. high [blue]) and the cumulative incidence of severe GvHD at D+30 (analyzed using Cox proportional hazards regression). D = day; GvHD = graft-versus-host disease. (**D**) Association between zonulin levels (low [red] vs. high [blue]) and the cumulative incidence of severe GvHD using ΔZonulin (D0 minus D−7; (analyzed using Cox proportional hazards regression). D = day; GvHD = graft-versus-host disease.

Table 3. Associations Between Zonulin and Clinical Outcomes.

	D−7		D0		D+30	
	HR (95% CI)	***p* Value**	**HR (95% CI)**	***p* Value**	**HR (95% CI)**	***p* Value**
GvHD	1.00 (1.00, 1.01) *	0.6	1.00 (1.00, 1.01) †	0.4	1.00 (0.99, 1.01)	0.7 Î
Severe GvHD	1.00 (0.98, 1.01) **	0.5	1.00 (0.99, 1.01) ††	0.6	0.99 (0.97, 1.00)	0.12 ÎÎ
Overall Survival	1.00 (0.99, 1.01) *	>0.9	1.00 (0.99, 1.01) †	>0.9	1.01 (0.99, 1.02)	0.5 Î
BSI	1.00 (0.99, 1.01) *	>0.9	1.00 (0.99, 1.01) †	0.8	1.00 (0.97, 1.03)	>0.9 ÎÎÎ

BSI = Bloodstream infection; CI = Confidence interval; D = Day; GvHD = Graft-versus-graft disease; HR = Hazard Ratio; * = 124 patients included in this analysis; ** = 35 patients included in this analysis; † = 129 patients included in this analysis; †† = 38 patients included in this analysis; Î = 77 patients included in this analysis; ÎÎ = 19 patients included in this analysis; ÎÎÎ = 82 patients included in this analysis.

2.4.2. Associations Between Zonulin and Overall Survival

Zonulin was not significantly associated with overall survival when analyzed as a categorical variable based on the median value (D−7: HR 1.26; 95% CI 0.58–2.72; $p = 0.6$; D0: HR 0.75; 95% CI 0.37–1.55; $p = 0.4$; D+30: HR 1.03; 95% CI 0.36–2.96; $p > 0.9$; ΔZonulin: HR 0.67; 95% CI 0.30–1.51; $p = 0.3$; see Figure 4). When analyzed as a continuous variable (see Table 3), zonulin levels were not significantly associated with overall survival at the evaluated time points.

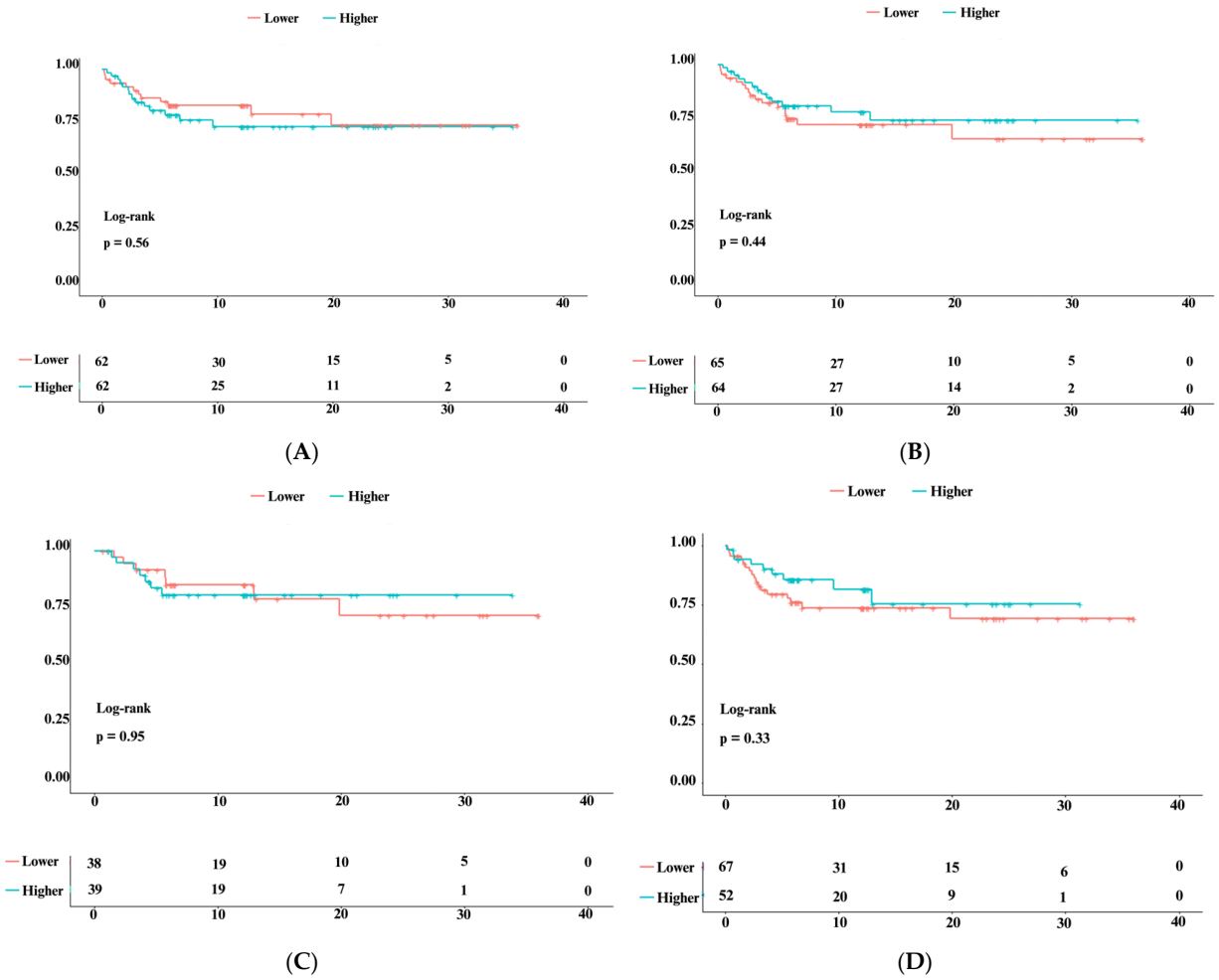


Figure 4. (**A**) Association between zonulin levels (low [red] vs. high [blue]) and overall survival at D−7 (analyzed using log-rank and Kaplan–Meier). D = day. (**B**) Association between zonulin levels (low [red] vs. high [blue]) and overall survival at D0 (analyzed using log-rank and Kaplan–Meier). D = day. (**C**) Association between zonulin levels (low [red] vs. high [blue]) and overall survival at D+30 (analyzed using log-rank and Kaplan–Meier). D = day. (**D**). Association between zonulin levels (low [red] vs. high [blue]) and overall survival using ΔZonulin (D0 minus D−7; (analyzed using log-rank and Kaplan–Meier). D = day.

2.4.3. Associations Between Zonulin and BSI

Similarly to the other clinical endpoints, there was no significant association between zonulin levels and BSI when patients were stratified as high versus low at any of the time points (D−7: OR 0.77; 95% CI 0.37–1.57; p = 0.5; D0: OR 1.09; 95% CI 0.54–2.21; p = 0.8; D+30: OR 1.64; 95% CI 0.25–13.4; p = 0.6; ΔZonulin: HR 0.61; 95% CI 0.29–1.29; p = 0.2). Zonulin was also not significantly associated with BSI when analyzed as a continuous variable (see Table 3).

3. Discussion

In this multicenter, prospective study evaluating the dynamics, predictors, and prognostic significance of zonulin in the allo-HSCT setting, we identified several major findings. First, zonulin levels remained consistently elevated throughout the allo-HSCT course. Second, no clinical or procedure-related characteristics appeared to be good predictors of increased zonulin levels. Finally, although zonulin levels were persistently elevated, they did not appear to be a useful prognostic marker.

An interesting finding of this study is that zonulin levels remained consistently elevated throughout the entire course of allo-HSCT, suggesting persistently gastrointestinal barrier damage. This is partially supported by prior studies [1–3,11,14,18–23]. Although intestinal permeability has been consistently reported to be increased in patients undergoing allo-HSCT, several studies have shown significant temporal dynamics [2,11,13,14,18,22,24–26]. For instance, in a study including 80 allo-HSCT patients, the lactulose:rhamnose (L:R) ratio was used to assess intestinal permeability at the following time points: baseline (preconditioning), D+7, and D+30 [18]. Compared with baseline, the L:R ratio significantly increased at D+7 (0.19 vs. 0.86; $p < 0.0001$) and at D+30 (0.19 vs. 0.40; $p = 0.014$) [18]. However, there was a significant decrease at D+30 compared with D+7 (0.86 vs. 0.40; $p = 0.014$) [18]. Similar findings were reported in other studies using the L:R ratio to evaluate intestinal permeability [13,24,25]. In contrast, conflicting findings in intestinal permeability dynamics were reported when biomarkers such as occludin and calprotectin were used as surrogate markers [1,11,18]. In a study including 31 allo-HSCT patients, calprotectin was measured at the following time points: D−7, D+7, D+14, and D+21 [1]. Calprotectin levels were significantly decreased compared with reference values, and no significant differences were observed among samples collected at different time points during the allo-HSCT course (D−7 vs. D+7: $p = 0.374$; D−7 vs. D+14; p value not reported; D−7 vs. D+21; $p = 0.451$) [1]. However, in a pediatric study, significant changes in calprotectin levels were observed during the allo-HSCT (Baseline (before allo-HSCT): 52.53 vs. D+90: 123 vs. D+180 90.05 ug/g; $p < 0.001$) [11]. Together with our findings, these studies highlight the challenges of assessing intestinal permeability during allo-HSCT.

These challenges are further compounded by evidence that distinct mechanisms may transiently contribute to increased intestinal permeability at different stages of the allo-HSCT course [21]. It is important to highlight that intestinal permeability is a complex concept that encompasses multiple gastrointestinal components, and each available methodology assesses only specific aspects of the intestinal barrier. In this context, the persistently elevated zonulin levels observed in our cohort may suggest sustained activation of the zonulin-mediated pathway throughout the allo-HSCT course, but not necessarily its direct relevance to the mechanisms underlying GvHD-related intestinal permeability. Alternatively, our findings may indicate that the zonulin pathway is already maximally activated prior to transplantation, thereby limiting its ability to provide incremental prognostic information. In contrast, a prior pediatric study including 100 allo-HSCT patients reported significant temporal changes in zonulin levels between pre-transplant and 3 months post-transplant (56.11 ± 15.43 vs. 90.69 ± 20.32; $p = 0.001$), as well as an association with GvHD severity ($p = 0.002$) [11]. Although these findings are not supported by our data, several important differences between the studies may account for these discrepancies. First, the pediatric study included only patients with complete longitudinal sampling at 3 and 6 months, which may have introduced a selection bias toward patients with fewer complications who survived longer after transplantation [11]. Second, patients with liver or renal dysfunction were excluded, potentially removing individuals with more severe allo-HSCT-related complications [11]. Third, the study population consisted exclusively of pediatric patients, whereas our cohort was predominantly adult [11]. Fourth, the authors did not provide detailed information regarding the zonulin assay used, and prior studies have demonstrated variability across different commercial kits [27–29]. Finally, there were differences in underlying diagnoses, with our cohort being largely composed of patients with malignant diseases, while the pediatric study primarily included non-malignant conditions such as hemophilia, immune disorders, and bone marrow failure syndromes [11]. Therefore, future studies should evaluate intestinal permeability during allo-HSCT using multiple complementary and validated methodologies. These complemen-

tary methodologies should aim to capture the distinct components of the intestinal barrier, including enterocyte mass (e.g., citrulline), enterocyte turnover (e.g., I-FABP [Intestinal Fatty Acid-Binding Protein]), paracellular permeability (e.g., zonulin, claudin, occludin, and L:R ratio), and transcellular transport (e.g., R:O [rhamnose:3-O-methyl-D-glucose] and X:O [xylose: 3-O-methyl-D-glucose] ratios). Only through such an approach will it be possible to accurately characterize the key pathways and mechanisms involved in intestinal barrier dysfunction in the allo-HSCT setting.

Another important finding of this study is that no patient- or procedure-related characteristics were identified as predictors of increased zonulin levels. Although serum zonulin has not been evaluated in prior adult allo-HSCT studies, predictors of increased intestinal permeability consistently reported in the literature include: (1) conditioning regimen [2,14,15,30], (2) dietary interventions [13,19,23], (3) intestinal microbiota [18,19], and (4) immune response [20,30,31]. Regarding the conditioning regimen, studies have shown that myeloablative conditioning, compared with other regimens, is associated with increased levels of 51Cr-EDTA (Chromium-51 labeled ethylenediaminetetraacetic acid) [2,14], and decreased levels of citrulline [30], and I-FABP [15]. Myeloablative conditioning, however, was not associated with changes in the L:R and L:M ratios [1,18]. Similarly, conflicting findings have been reported regarding the intestinal microbiota [18,19]. While both microbiota diversity indices and microbiota composition have been associated with the L:R ratio [18], no significant associations were observed when analyzing I-FABP, LBP (Lipopolysaccharide-binding protein), and sCD14 (Soluble cluster of differentiation 14) [19]. Taken together, these findings further highlight the complexity of assessing intestinal permeability in the allo-HSCT setting. Because each methodology evaluates specific components of the gastrointestinal barrier, the identified predictors may differ across approaches. Therefore, future studies should not only apply multiple complementary methodologies but also comprehensively assess potential predictive factors. Identifying predictors of increased intestinal permeability is clinically relevant, as these factors may represent therapeutic targets for interventions aimed at modulating intestinal barrier function.

The final major finding of this study is that zonulin levels were not significantly associated with overall survival, aGvHD, or BSI. The consistent observation of hazard ratios close to 1 with relatively narrow 95% confidence intervals, combined with our relatively large cohort (n = 140), supports the interpretation that this represents a true negative finding rather than the result of insufficient statistical power. This is partially supported by prior studies, which have reported conflicting results regarding the prognostic significance of intestinal permeability markers [1–3,11,15,19,22–25,31–34]. For instance, while intestinal permeability markers were not associated with overall survival [18,19,32,34], significant associations have been reported when analyzing aGvHD [1–3,23]. Although hypotheses exist regarding translocation of the intestinal microbiota as a source of BSI in the allo-HSCT setting, studies have consistently reported non-significant associations with several distinct permeability markers, including the L:R ratio, L:M ratio, calprotectin, beta-defensin-2, haptoglobin genotype, and LBP [1,3,18,31,34]. Interestingly, in a study evaluating the prognostic significance of serum zonulin in pediatric patients undergoing allo-HSCT, increased zonulin levels were associated with GvHD severity (110.05 vs. 62.33 ng/mL; p = 0.002), diarrhea (100.23 vs. 72.22 ng/mL; p = 0.036), and mucositis (105.81 vs. 66.22 ng/mL; p = 0.029) [11]. Overall, our findings, together with prior studies, suggest that the prognostic significance of intestinal permeability during the allo-HSCT course remains inconclusive. Several methodological limitations, including small sample sizes and heterogeneity in permeability assessment methods, may contribute to these inconsistent results. Therefore, future studies should evaluate intestinal permeability in larger cohorts using standardized methodologies.

The primary strength of this study is the longitudinal assessment of zonulin levels in a large cohort of patients undergoing allo-HSCT, particularly when compared with prior studies of intestinal permeability in this setting [1,2,14,18,20–22,35]. Additionally, to our knowledge, this is the first study to systematically evaluate serum zonulin dynamics and prognostic significance in the adult allo-HSCT setting. This study, however, has some limitations. First, prior studies have demonstrated concerns regarding the sensitivity and specificity of commercially available zonulin kits [27–29]. Evidence suggests that these assays may not specifically measure zonulin (prehaptoglobin-2) or correlate with haptoglobin genotypes, but instead detect a group of structurally related proteins, including complement components such as C3 and properdin [27–29]. However, the zonulin ELISA kit used in our study reports no significant cross-reactivity with structurally related proteins according to the manufacturer's specifications. Nonetheless, independent validation studies assessing its analytical specificity are still needed. Second, although predefined time points for sample collection were established, there was a significant decrease in the number of blood samples collected after D+30. This reduction may be attributable to several factors, including withdrawal from follow-up and mortality. This reduction may introduce attrition bias and substantially decrease the number of available samples at later timepoints, thereby limiting our ability to detect significant longitudinal changes in zonulin levels and to assess the prognostic significance of late timepoint samples for allo-HSCT outcomes. Finally, we did not validate zonulin levels using the gold-standard method for assessing intestinal permeability (namely Ussing chamber assays and/or probe-based methodologies). Notwithstanding these limitations, this study provides novel insights into the potential role of zonulin in the allo-HSCT setting. Future studies should simultaneously evaluate multiple complementary measures of intestinal permeability to comprehensively assess the different components of the intestinal barrier. Additionally, further studies are needed to validate zonulin assays in the context of allo-HSCT. Only with such validation studies can accurate conclusions be drawn regarding the prognostic significance of zonulin in the allo-HSCT setting.

4. Materials and Methods

4.1. Study Design and Ethical Aspects

This was a prospective, observational, multicenter cohort study of patients undergoing allo-HSCT. The study was approved on 1 December 2021, by the Research Ethics Committee of São Paulo State University (protocol number 5.138.190/2021) and was conducted in accordance with the Declaration of Helsinki. Adult participants provided written informed consent at the beginning of the study. For pediatric patients, both the patient and their legal guardian agreed to participate and provided signed informed consent forms. The inclusion criteria were patients aged $\geq$12 years undergoing allo-HSCT who provided at least one blood sample during the 6-month follow-up period. Transplants from related donors (including matched related donors and haploidentical donors) as well as unrelated donors (including matched and mismatched unrelated donors) were included. The exclusion criteria were: (1) patients undergoing autologous HSCT or umbilical cord blood transplantation, and (2) patients without adequate clinical follow-up or biological samples. Fifteen healthy adult controls without chronic inflammatory diseases or recent use of antibiotics, anti-inflammatory drugs, or immunosuppressants also signed informed consent forms and agreed to participate in the study.

4.2. Sample Collection and Zonulin Measurement

Blood samples (5 mL) were collected from participants into K2-EDTA tubes at four different Brazilian hospitals (Hospital de Base of the Fundação Faculdade Regional de

Medicina [HB-FUNFARME], Hospital Amaral Carvalho [HAC], Hospital de Câncer de Barretos [HCB], and Hospital Beneficência Portuguesa de São Paulo [BP]). Plasma was separated by centrifugation at 1372× *g* for 10 min and temporarily stored at −20 °C until transport to the central laboratory, where it was stored at −80 °C until analysis. Plasma zonulin concentrations were measured using a commercial enzyme-linked immuno-sorbent assay (Human Zonulin ELISA Kit, Elabscience, Houston, TX, USA), according to the manufacturer's instructions. Although limitations in zonulin specificity have been described for some commercial ELISA assays, the manufacturer of the ELISA used in our study reports no significant cross-reactivity with structurally related proteins [27,28]. Optical density (OD) was read at 450 nm using a spectrophotometer. Calibration curves were constructed in Excel using the equation y = ax + b, in which x and y represented OD and concentration, respectively. Zonulin concentrations were calculated by converting OD values into ng/mL. Samples were collected longitudinally at seven time points: prior to the conditioning regimen (D−7), on the day of stem cell infusion (D0), 30 days after transplantation (D+30), D+60, D+90, D+180, and at the time of acute graft-versus-host disease (aGvHD) diagnosis. Although efforts were made to collect samples at these exact time points, some variability occurred due to rescheduling of follow-up visits and intercurrent clinical events.

4.3. Clinical Data and Outcomes

Study data were collected and managed using REDCap (Research Electronic Data Capture) electronic data capture tools hosted at HCB [36,37]. REDCap is a secure, web-based software platform designed to support data capture for research studies, providing (1) an intuitive interface for validated data capture; (2) audit trails for tracking data manipulation and export procedures; (3) automated export procedures for seamless data downloads to common statistical packages; and (4) procedures for data integration and interoperability with external sources [36,37]. Variables collected included detailed demographic characteristics and transplant-related data. Outcomes of interest were overall survival, aGvHD (cumulative incidence), and BSI. Acute GvHD was diagnosed clinically, confirmed histopathologically by biopsy whenever possible, and graded according to the MAGIC criteria [38]. As in previous studies, the onset of aGvHD was defined based on the appearance of clinical symptoms (clinical suspicion), as determined by transplant specialists based on manifestations commonly associated with acute GvHD, including skin rash, nausea, vomiting, diarrhea, and jaundice [39,40]. BSI was defined by the presence of positive blood cultures within the first 90 days after allo-HSCT.

4.4. Statistical Analysis

We initially generated descriptive statistics stratified by center. To evaluate the longitudinal dynamics of zonulin levels during allo-HSCT, paired Wilcoxon tests were used. To identify predictors of increased zonulin levels, logistic regression analysis was performed. Based on prior literature, the following potential predictors were included: (1) demographic characteristics (participating center, age, weight, height, and sex); (2) history of previous HSCT; (3) use of total body irradiation (TBI) in the conditioning regimen; (4) type of conditioning (myeloablative, non-myeloablative, or reduced-intensity); (5) underlying disease (leukemias versus other indications); and (6) use of antibiotics [1,18,19]. Antibiotic data was available in two centers (HB-FUNFARME and BP).

To evaluate the association between zonulin levels and clinical outcomes, Kaplan–Meier analysis and the log-rank test were used for overall survival, and Cox proportional hazards regression was used to assess the cumulative incidence of aGvHD. For GvHD, given our interest in evaluating zonulin as a predictive biomarker, we considered only events occurring after sample collection. Accordingly, for analyses of samples collected at D+30, we

excluded patients who developed GvHD prior to that time point. To assess the relationship between zonulin levels and BSI, we used logistic regression models. For all the outcomes, these associations were analyzed using unadjusted models and considering zonulin both as a continuous and as a categorical variable. When analyzed as a categorical variable, zonulin levels were dichotomized based on the median value at each time point, classifying patients into high and low groups. Additionally, given our interest in evaluating zonulin as a prognostic biomarker, we also assessed predictive performance (sensitivity, specificity, and predictive values) when zonulin was analyzed as a categorical variable. Given our goal of evaluating zonulin as a predictive biomarker, we assessed only zonulin levels at D−7, D0, and D+30. We also evaluated ΔZonulin (the difference between D0 and D−7). All statistical analyses were performed using R software (version 4.5.3), and p values < 0.05 were considered statistically significant.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data associated with this study will not be made publicly available. However, data associated with this project may be shared upon specific request in a deidentified manner and in accordance with appropriate data-sharing requirements.

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Abbreviations

The following abbreviations are used in this manuscript:

aGvHD	Acute Graft-versus-Host Disease
allo-HSCT	Allogeneic Hematopoietic Stem Cell Transplantation
ATB	Antibiotic
BMI	Body Mass Index
BP	Hospital Beneficência Portuguesa de São Paulo
BSI	Bloodstream Infection
CI	Confidence Interval
D	Day
ELISA	Enzyme-Linked Immunosorbent Assay
GI-GvHD	Gastrointestinal Graft-versus-Host Disease
GvHD	Graft-versus-Host Disease
HAC	Hospital Amaral Carvalho
HB-FUNFARME	Hospital de Base da Fundação Faculdade Regional de Medicina
HCB	Hospital de Câncer de Barretos
HLA	Human Leukocyte Antigens
HR	Hazard Ratio
HSCT	Hematopoietic Stem Cell Transplantation
I-FABP	Intestinal Fatty Acid-Binding Protein
K2-EDTA	Dipotassium Ethylenediaminetetraacetic Acid
LBP	Lipopolysaccharide-Binding Protein
L:M ratio	Lactulose:Mannitol Ratio
L:R ratio	Lactulose:Rhamnose Ratio
MAGIC	Mount Sinai Acute GVHD International Consortium
MMUD	Mismatched Unrelated Donor
MRD	Matched Related Donor
MUD	Matched Unrelated Donor
N	Number
N/A	Not Applicable
NR	Not Reported
OD	Optical Density
OR	Odds Ratio
REDCap	Research Electronic Data Capture
sCD14	Soluble CD14
SD	Standard Deviation
TBI	Total Body Irradiation

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Supplementary Tables

Supplementary Table S1 – Number of samples per time point

Center	D-7	D0	D30	D60	D90	D180	aGvHD	Total
HCB	10	10	8	6	6	2	2	44
HAC	47	47	30	15	11	4	0	154
HB-FUNFARME	29	30	23	16	10	8	7	123
BP	38	42	21	22	16	0	17	156
Total	124	129	82	59	43	14	26	477

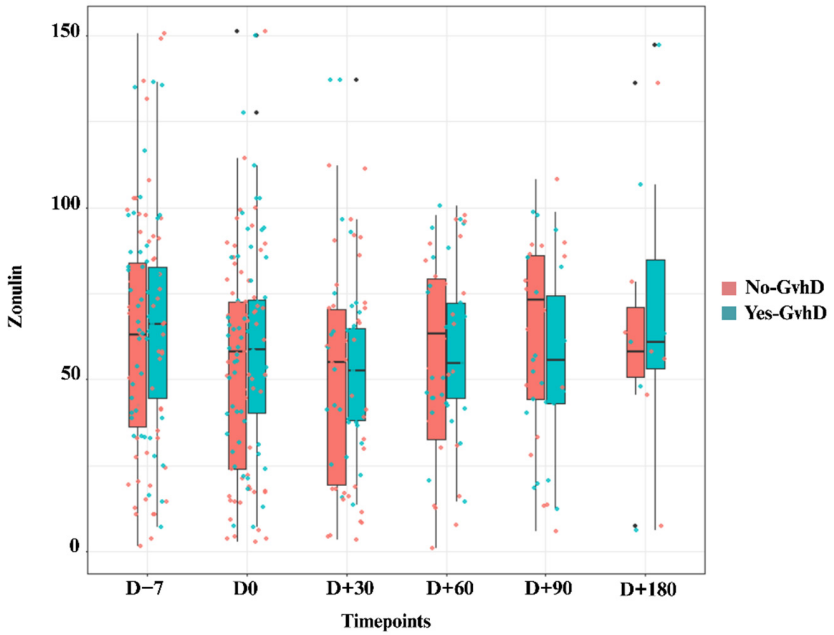
aGvHD: acute graft-versus-host-disease; BP = Hospital Beneficência Portuguesa de São Paulo; D = day; HCB = Hospital de Câncer de Barretos; HAC = Hospital Amaral Carvalho; HB-FUNFARME = Hospital de Base of the Fundação Faculdade Regional de Medicina.

Supplementary Table S2 – Diagnostic Performance of Zonulin for Allo-HSCT–Related Outcomes

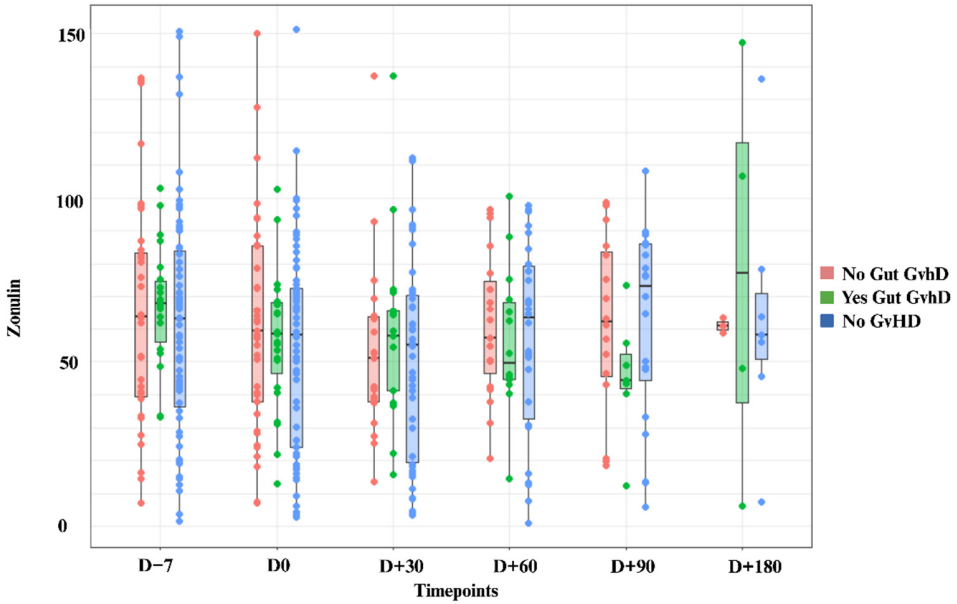
	GvHD			Severe GvHD			Overall Survival			BSI		
	D−7	D0	D+30	D−7	D0	D+30	D−7	D0	D+30	D−7	D0	D+30
Sensitivity	0.526	0.500	0.484	0.600	0.605	0.579	0.538	0.433	0.500	0.462	0.509	0.600
Specificity	0.522	0.507	0.478	0.539	0.549	0.517	0.510	0.485	0.492	0.472	0.513	0.523
Positive Predictive Value	0.484	0.469	0.385	0.339	0.359	0.282	0.226	0.203	0.179	0.387	0.422	0.125
Negative Predictive Value	0.565	0.538	0.579	0.774	0.769	0.789	0.806	0.738	0.816	0.548	0.600	0.920

Allo-HSCT = Allogeneic hematopoietic stem cell transplantation; BSI = Bloodstream infection; GvHD = Graft-versus-host disease

Supplementary Figures



Supplementary Figure S1. (A)



Supplementary Figure S1. (B)

Supplementary Figure S1. (A) Temporal dynamics of zonulin throughout the allo-HSCT course stratified according to the presence of GvHD. **(B)** Temporal dynamics of zonulin throughout the allo-HSCT course stratified according to the presence of gut GvHD. D = day; GvHD = graft-versus-host disease.

5.3.3. Manuscrito III – Artigo Original – Crosstalk Microbiota, Permeabilidade e Citocinas

The Dynamics and Crosstalk Between the Gut Microbiota, Intestinal Permeability and Cytokines in Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation

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ABSTRACT

Background: In the allogeneic hematopoietic stem cell transplantation (allo-HSCT) setting, a better understanding of the gut barrier–immune axis may improve prognostic stratification and identify novel therapeutic targets. Therefore, the objective of this study was to investigate the correlations among gut microbiota, intestinal permeability, and cytokine profiles in a cohort of Brazilian patients undergoing allo-HSCT.

Study Design and Methods: This multicenter prospective cohort study included patients undergoing allo-HSCT at four Brazilian institutions. Patients aged ≥12 years were eligible if they provided at least one blood or stool sample during the transplantation course. Samples were collected longitudinally at six pre-determined timepoints. Gut microbiota composition, with a particular focus on bacterial genera known to include short-chain fatty acid (SCFA)-producing species, was assessed by 16S rRNA gene sequencing using the Illumina platform. Intestinal permeability was evaluated by measuring serum zonulin levels using an enzyme-linked immunosorbent assay (ELISA). Cytokine concentrations were quantified using a cytometric bead array. First, we evaluated the longitudinal dynamics of each component throughout the allo-HSCT course. We then assessed the correlations among gut microbiota composition, serum zonulin, and cytokine levels using Spearman's rank correlation coefficients.

Results: A total of 169 patients were included in the gut microbiota cohort, 148 in the cytokine cohort, and 140 in the zonulin cohort. Compared with healthy controls, patients undergoing allo-HSCT exhibited significantly lower gut microbial alpha diversity and higher serum zonulin levels. Throughout the allo-HSCT course, alpha diversity and serum zonulin levels remained relatively stable, whereas cytokine levels changed significantly over time. Correlation analysis revealed several significant associations between SCFA-producing bacterial genera and both

cytokine and zonulin levels, including negative correlations between *Bifidobacterium* and IL-6 and IFN-γ levels, and between *Clostridia* and serum zonulin levels.

Conclusion: This study provides novel insights into the interactions within the gut barrier–immune axis in the allo-HSCT setting. Future studies incorporating additional markers of intestinal permeability, immune reconstitution, and microbiome function are needed to further characterize this axis and determine its impact on clinical outcomes following allo-HSCT.

Keywords: Intestinal Barrier Function; Gastrointestinal Microbiome; Immune Reconstitution; Stem Cell Transplantation; Cytokines.

1. INTRODUCTION

An important prognostic and therapeutic variable in the allogeneic hematopoietic stem cell transplantation (allo-HSCT) setting that has gained increasing attention is the gut barrier–immune axis.[1] This axis refers to the bidirectional interaction between the intestinal microbiota, the intestinal epithelial barrier, and the host immune system.[1] Through this complex interplay, microbial composition and function can influence intestinal barrier integrity, and immune homeostasis, which are particularly relevant in the allo-HSCT setting.[1] In the allo-HSCT setting, immune reconstitution is a critical process that requires careful modulation to achieve an optimal balance between effective immune surveillance and immune tolerance.[2] An adequate immune response is necessary to protect the host against infections and promote the graft-versus-leukemia (GVL) effect; however, impaired immune reconstitution may contribute to the development of graft-versus-host disease (GVHD).[2,3]

Prior studies have highlighted the potential impact of each component of the gut barrier–immune axis in the allo-HSCT setting.[3–6] For instance, reduced intestinal microbiota diversity has been associated with an increased risk of GVHD and decreased overall survival.[7] Similarly, increased intestinal permeability has been linked to the development of severe GVHD and higher non-relapse mortality.[8,9] Furthermore, several markers of immune reconstitution following allo-HSCT have been associated with reduced risks of infections and disease relapse.[2,3] Although evidence exists regarding the individual contributions of the microbiota, intestinal barrier, and immune system after allo-HSCT, the interplay among these components and the integrated role of the gut barrier–immune axis remains poorly understood.[1]

In our previous studies, we investigated gut microbiota domination events and intestinal permeability as individual components in patients undergoing allo-HSCT.[4,10] Building on these

findings, we performed a longitudinal analysis integrating these parameters with cytokine profiles to better understand the interplay among gut microbiota, intestinal permeability, and cytokines. To date, only a limited number of studies have longitudinally evaluated the relationship between these parameters.[1] Therefore, our aim was to investigate the relationship between gut microbiota, intestinal permeability, and cytokines, in order to identify potential mechanisms through which the microbiota may influence outcomes after allo-HSCT.

2. MATERIAL AND METHODS

2.1. STUDY DESIGN, ETHICAL ASPECTS AND SAMPLE COLLECTION

This was a prospective, multicenter, observational cohort study of patients undergoing allo-HSCT between 2021 and 2025. The study was reviewed and approved by the Research Ethics Committee of São Paulo State University (process number 5.138.190/2021) and conducted in accordance with the Declaration of Helsinki. Eligible patients were those undergoing allo-HSCT at participating centers who provided at least one stool and/or blood sample during the study period and had clinical data available in REDCap. Patients were excluded if they did not provide any biological samples within the 6-month follow-up period or if they underwent cord blood or autologous transplantation.

Biological samples were collected at the following pre-defined time points: D−7 (baseline), D0 (day of stem cell infusion), D+30, D+60, D+90, D+180, and at the time of suspected acute GvHD. Samples were temporarily stored at −20 °C at each participating center prior to transfer to the central laboratory, where they were stored at −80 °C. Efforts were made to collect both samples (stool and blood) at these predefined time points; however, some variability occurred due to hospital discharge, timing and frequency of bowel movements, and acute clinical

complications. The overall cohort consisted of patients who had at least one of the three laboratory assessments performed (intestinal microbiota, immune markers, or intestinal permeability). To be included in a specific sub cohort, patients were required to have the corresponding parameter assessed.

2.2. BLOOD SAMPLES (CYTOKINES AND ZONULIN)

K2-EDTA tubes were used to collect blood samples from patients undergoing allo-HSCT. The plasma was isolated by centrifugation at 1,372 × g for 10 min and temporarily stored at −20 °C until transport to the central laboratory, where it was stored at −80 °C until analysis. Cytokine concentrations were determined using a cytometric bead array (Human Th1/Th2/Th17 Kit, BD Biosciences, San Jose, CA, USA).[11] Levels of interleukin (IL)-2, IL-4, IL-6, IL-10, IL-17A, interferon-gamma (IFN-γ), and tumor necrosis factor (TNF) were quantified by flow cytometry (FACSCanto™ II, BD Biosciences, Franklin Lakes, NJ, USA).[11] Data acquisition and analysis were performed using BD FCAP Array™ v3 software, and results were expressed in pg/mL.[11] Plasma zonulin levels were assessed by enzyme-linked immunosorbent assay (Human Zonulin ELISA Kit, Elabscience, USA), according to the manufacturer's protocol.[4] Optical density was measured at 450 nm, and standard curves were generated using a linear equation ($y = ax + b$), where OD values were converted into concentrations expressed in ng/mL.

2.3. STOOL SAMPLES (DNA EXTRACTION AND 16S SEQUENCING)

Stool samples were collected from patients undergoing allo-HSCT for gut microbiota profiling. Microbial DNA was extracted from approximately 200 mg of fecal material using the QIAamp Fast DNA Stool Mini Kit (Qiagen, CA, USA), following the manufacturer's instructions, and quantified with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific,

Waltham, MA, USA).[10] The V3–V4 hypervariable regions of the 16S rRNA gene were amplified using a two-step PCR approach with previously described primers (341F and 805R).[10] Amplification was performed using 2X Platinum SuperFi II PCR Master Mix (Thermo Fisher Scientific) under the following conditions: initial denaturation at 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, 55 °C for 10 s, and 72 °C for 30 s.[10] Sequencing was carried out on the Illumina NextSeq 1000 platform (Illumina, San Diego, CA, USA), generating a total of 108,251,088 reads (mean ~217,371 reads per sample). Adapter trimming was performed using Cutadapt (v2.6), followed by quality control, denoising, chimera removal, and amplicon sequence variant (ASV) inference with DADA2 (v1.26.0). After filtering, 59,994,735 reads (mean ~120,471 reads per sample) were retained for downstream analyses. Taxonomic assignment was conducted using a naive Bayes classifier implemented in the QIIME 2 platform (q2-feature-classifier plugin), trained on the SILVA SSU database (v138.1, 99% similarity). Relative abundances were calculated at the ASV level and subsequently aggregated for analysis. Microbial community composition was visualized using the taxa plugin (barplot function). Alpha- and beta-diversity metrics, as well as statistical comparisons across study groups, were computed using the QIIME 2 diversity plugin. To account for differences in sequencing depth, samples were rarefied to 30,000 reads prior to diversity analyses.

2.4. STATISTICAL ANALYSIS

Descriptive statistics were initially generated and stratified according to data availability for each of the three domains: cytokines, gut microbiota, and intestinal permeability. To provide a comprehensive overview and assess the interplay among cytokines, gut microbiota, and intestinal permeability, we first characterized their individual dynamics.[1] To evaluate this longitudinal dynamics we used Dunn’s test (with false discovery rate (FDR) correction) for cytokines, paired

Wilcoxon tests for zonulin and Kruskal-Wallis (adjusted for multiple comparisoms) for intestinal microbiota. While our previous studies had described zonulin dynamics and explored gut microbiota domination events, the present study expands these analyses by incorporating additional samples and an assessment of short-chain fatty acid-producing bacteria (SCFA).[4,10] SCFAs are key metabolites produced by certain intestinal microbiota bacteria that promote gut homeostasis, maintain intestinal permeability and regulate the immune system.[7] Notably, this is the first study to systematically evaluate cytokine dynamics within our cohort. To assess the correlations between the intestinal microbiota, cytokines, and intestinal permeability, heatmaps based on Spearman's correlation coefficients was generated. The heatmaps were generated based on the timing of sample collection, from D−7 to D+180. For the heatmaps, we included the microbial diversity index, the relative abundances of known SCFA-producing bacteria, the relative abundances of genera previously identified as drivers of intestinal domination events in our prior study,[10] as well as zonulin and cytokine levels. Statistical analyses were performed using R software (version 4.5.3), and p values < 0.05 were considered statistically significant.

3. RESULTS

3.1. DEMOGRAPHIC CHARACTERISTICS AND CLINICAL OUTCOMES

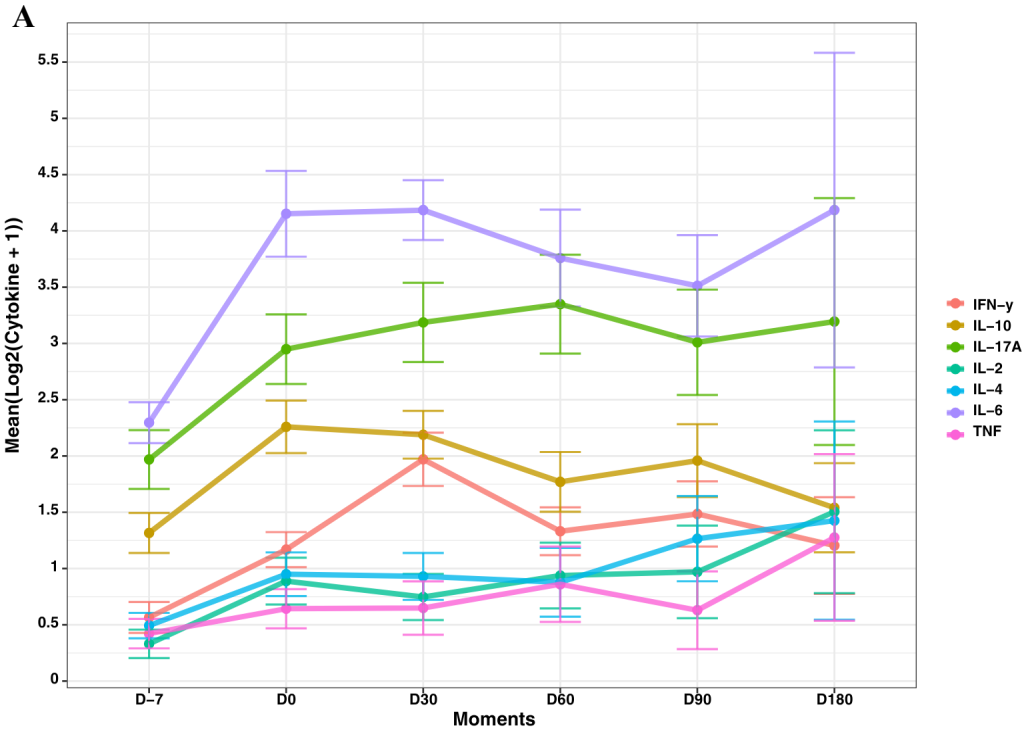
Demographic and clinical characteristics are summarized in **Table 1**. Overall, 169 patients were included in the intestinal microbiota cohort, 148 in the immune marker cohort, and 140 in the intestinal permeability cohort. Several patients had overlapping sample collections and were therefore included in more than one sub cohort. Specifically, 135 had both intestinal microbiota and immune marker data, 131 had intestinal microbiota and zonulin data, 132 had immune marker and zonulin data, and 129 had data available for all three features analyzed.

Table 1. Baseline Demographic and Clinical Characteristics of the Overall Cohort and Sub Cohorts			
Variable	**Intestinal Microbiota (N = 169)**	**Immune Markers (N = 148)**	**Intestinal Permeability (N = 140)**
Age			
Mean (SD)	42.4 ± 15.7	44.6 ± 16.0	42 ± 16
Median (Min-Max)	41.0 (12.0 – 73.0)	43.6 (14.0–75.5)	41 (12–73)
Weight (kg)			
Mean (SD)	75.0 ± 17.0	75.1 ± 17.0	75 ± 16
Median (Min-Max)	73.0 (43.0 – 131.4)	73.4 (23.0–130.0)	73 (43–130)
Height (cm)			
Mean (SD)	167.9 ± 10.2	168.0 ± 11.0	168 ± 10
Median (Min-Max)	167 (144 – 195)	168.0 (124–195)	168 (144–195)
Body Mass Index			
Mean (SD)	26.48 ± 5.25	26.5 ± 5.2	26.5 ± 5.0
Median (Min-Max)	25.26 (15.8 – 48.3)	25.6 (15.0 – 41.4)	25.8 (15.8 – 40.7)
Biological Sex			
Male	92 (54.4%)	84 (56.8%)	79 (56.4%)
Female	77 (45.6%)	64 (43.2%)	61 (43.6%)
Prior Allo-HSCT	13 (7.7%)	11 (7.4%)	10 (7.1%)
Underlying Disease			
Acute Leukemia	110 (65.1%)	97 (65.5%)	92 (65.8%)
Lymphoma	8 (4.7%)	6 (4.1%)	7 (5.0%)
Aplastic Anemia	11 (6.5%)	11 (7.4%)	10 (7.1%)
Sickle Cell Disease	4 (2.4%)	4 (2.7%)	4 (2.9%)
Other	36 (21.3%)	30 (20.3%)	27 (19.3%)
Donor Type			
Matched Related	52 (30.8%)	45 (30.4%)	42 (30.0%)
Matched Unrelated	20 (11.8%)	20 (13.5%)	17 (12.1%)
Mismatched Related	4 (2.4%)	6 (4.1%)	5 (3.6%)
Haploidentical	83 (49.1%)	72 (48.6%)	71 (50.7%)
Mismatched Unrelated	8 (4.7%)	5 (3.4%)	5 (3.6%)
Donor Sex			
Male	103 (60.9%)	95 (64.2%)	90 (64.3%)
Female	66 (39.1%)	52 (35.1%)	50 (35.7%)
Intensity of Conditioning Regimen			
Ablative	57 (33.7%)	52 (35.1%)	51 (36.4%)
Reduced Intensity	79 (46.7%)	71 (48.0%)	68 (48.6%)
Nonmyeloablative	28 (16.6%)	24 (16.2%)	21 (15.0%)
allo-HSCT, allogeneic hematopoietic stem cell transplantation; HCT-CI, Hematopoietic Cell Transplantation–Comorbidity Index; IQR, interquartile range; SD, standard deviation; TBI, total body irradiation; N = Number of patients.			

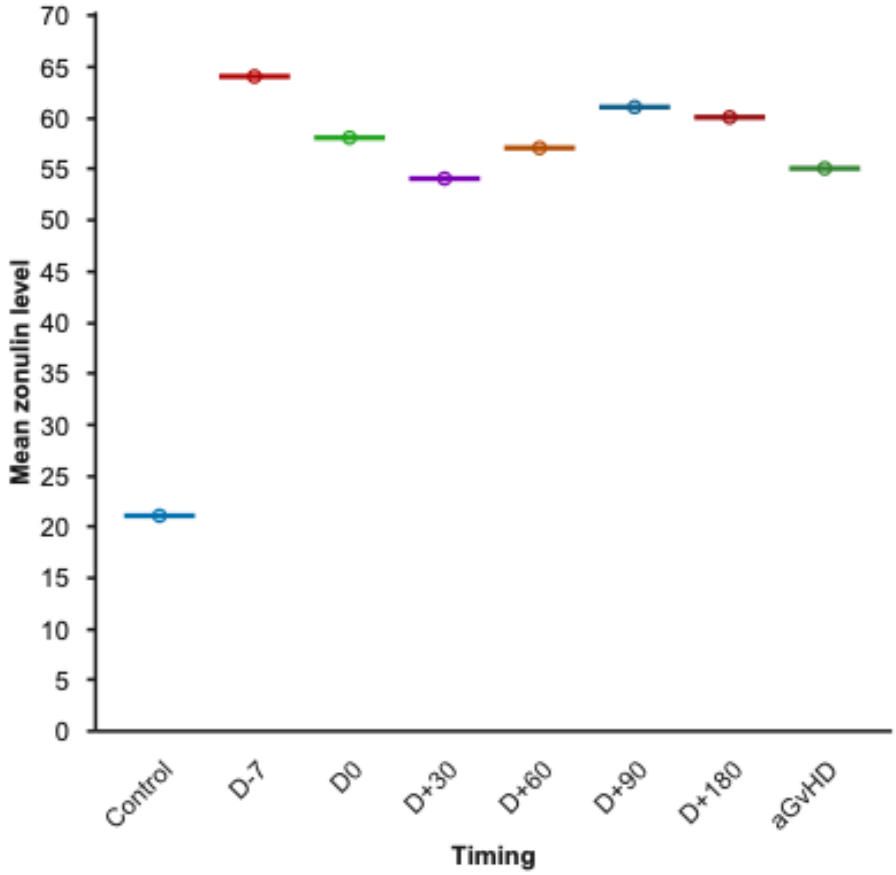
3.2. LONGITUDINAL CHANGES IN INTESTINAL MICROBIOTA, INTESTINAL PERMEABILITY, AND CYTOKINES

To provide a comprehensive overview of the dynamics and to assess the relationships among these components, we first characterized each parameter separately. An overview of cytokines, zonulin, and intestinal microbiota diversity dynamics is provided in **Figure 1A-1C**. A detailed description of the dynamics of each cytokine can be found in **Supplementary Figure 1A-G and Supplementary Figure 2.**

A


Mean(Log2(Cytokine + 1))
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
D−7
D0
D30
D60
D90
D180
Moments
IFN−y
IL−10
IL−17A
IL−2
IL−4
IL−6
TNF

B


Mean zonulin level
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
Control
D-7
D0
D+30
D+60
D+90
D+180
aGvHD
Timing

C

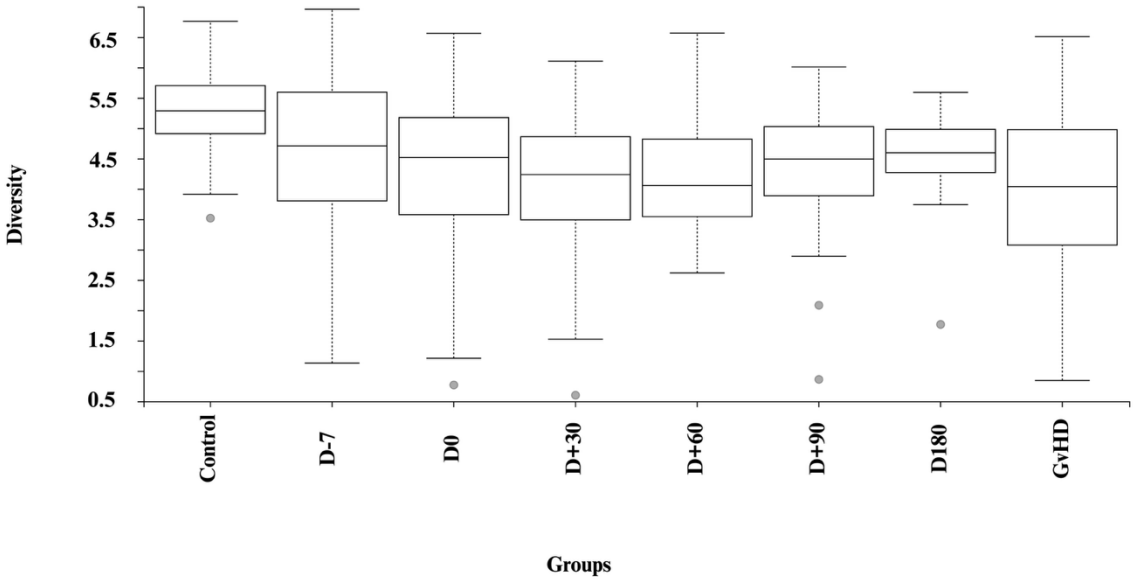


Figure 1. Overview of cytokine, zonulin, and intestinal diversity dynamics during allo-HSCT. (A) Cytokine dynamics over the allo-HSCT course. (B) Zonulin dynamics over the allo-HSCT course. (C) Intestinal diversity over the allo-HSCT course. D = day; IL = interleukin; TNF = tumor necrosis factor; IFN = interferon.

We observed significant temporal changes in cytokine levels throughout the allo-HSCT course for all cytokines except TNF, with most differences occurring relative to baseline (D−7). IFN-γ levels increased from 0.19 at D−7 to 1.12 at D0, 1.84 at D+30, 1.47 at D+60, and 1.49 at D+90 (all $p < 0.01$ vs. D−7), with additional differences between D+30 and D0 ($p < 0.01$), D+60 ($p < 0.01$), and D+90 ($p < 0.05$). IL-10 levels similarly increased from 1.09 at D−7 to 1.81 at D0, 1.84 at D+30, and 1.70 at D+90 ($p < 0.05$–0.01 vs. D−7), while IL-17A rose from 2.04 at baseline to 3.13 at D0, 3.29 at D+30, 3.35 at D+60, and 3.70 at D+90 (all $p < 0.01$). IL-2 and IL-4 levels also increased over time, although with more variable dynamics; IL-2 showed significant differences at D0 ($p < 0.01$) and D+30 ($p < 0.05$) compared to baseline, whereas IL-4 increased from 0.00 at D−7 to 0.71 at D0, 0.84 at D+30, and peaked at 1.10 at D+90 ($p < 0.01$ for D0 and D+90 vs. D−7; $p < 0.05$ for D+30 and D+180). Finally, IL-6 levels increased from 2.16 at D−7 to 3.72 at D0, peaking at 4.14 at D+30, followed by a gradual decline at D+60 (3.71), D+90 (3.31),

and D+180 (3.63), remaining significantly elevated compared to baseline at all post-transplant time points ($p < 0.01$).

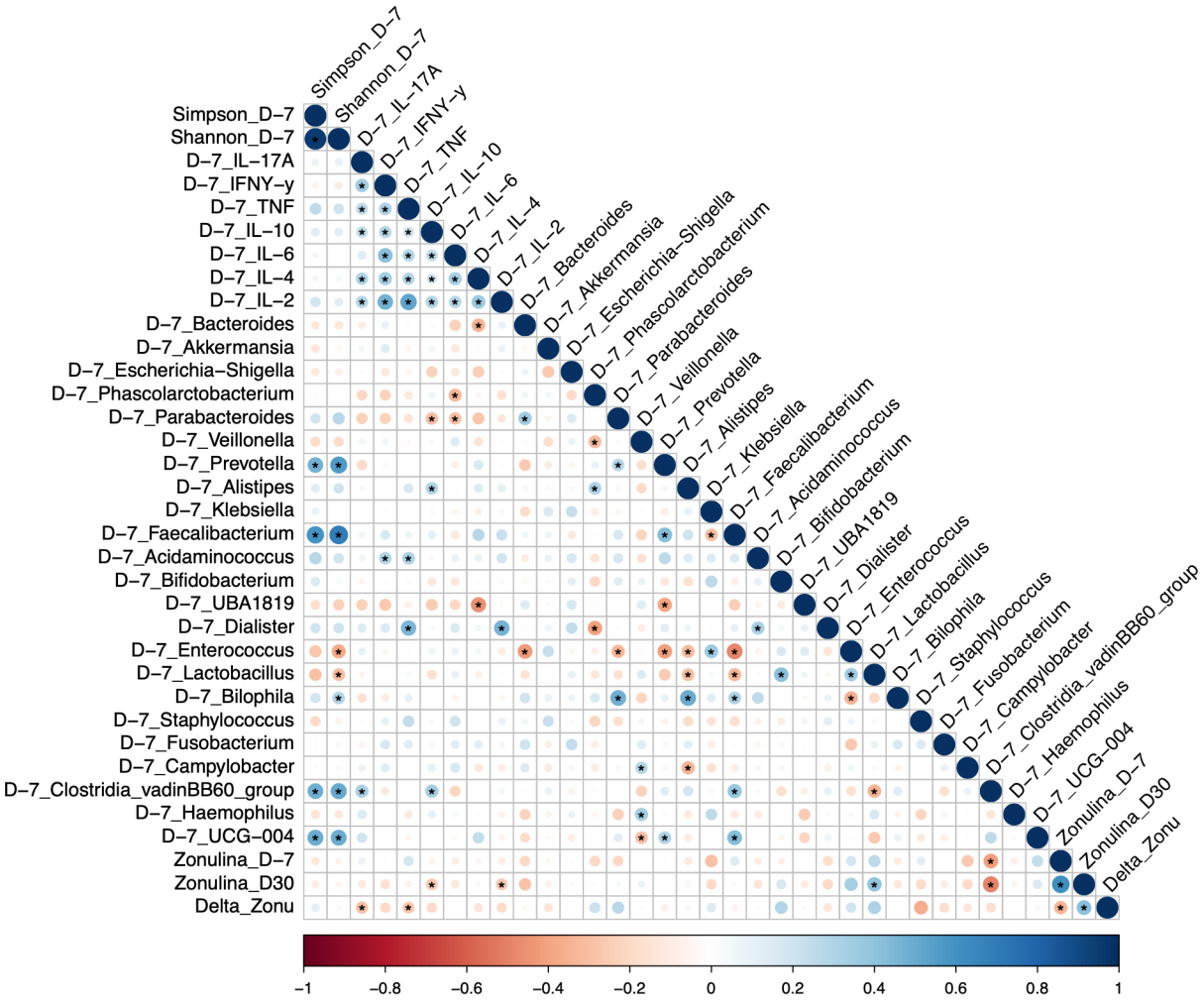
Zonulin levels were significantly increased in all timepoints over the allo-HSCT course when compared with healthy controls (see **Figure 1B**).[4] However, no significant dynamics between allo-HSCT timepoints were identified (see our prior publication for details).[4] Regarding gut microbiota diversity (see **Figure 1C**), we observed significant temporal changes when comparing healthy controls with allo-HSCT recipients and when comparing pre-transplant (D−7) samples with subsequent post-transplant time points. Compared with healthy controls, patients exhibited similar microbial diversity at D−7 ($q = 0.18$), but significantly lower diversity from D0 through D+90 ($q = 0.02$, $q = 0.004$, $q=0.004$, $q=0.02$; respectively), with recovery to baseline levels around D+180 ($q = 0.07$). Longitudinally, microbial diversity at D−7 was significantly higher than at D+30 ($q = 0.01$) and D+60 ($q = 0.02$). No other pairwise comparisons reached statistical significance.

3.3. CORRELATIONS BETWEEN INTESTINAL MICROBIOTA, INTESTINAL PERMEABILITY, AND CYTOKINES

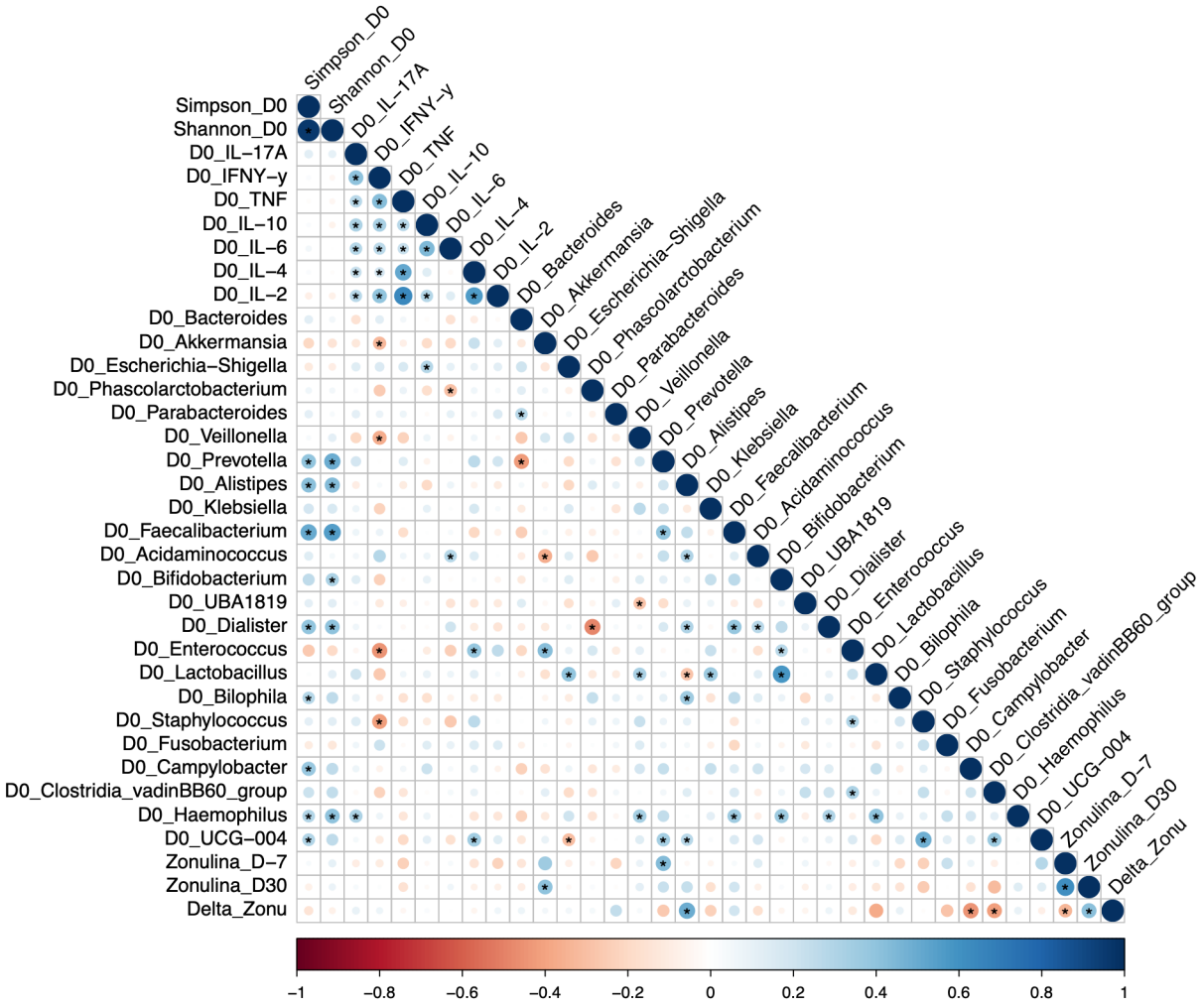
Our correlation analysis identified several significant correlations among intestinal microbiota features, zonulin, and cytokine levels throughout the allo-HSCT journey (see **Figure 2A-F**). When analyzing samples from D-7, negative correlations were observed between *Bacteroides* abundance and IL-4 levels, *Phascolarctobacterium* abundance and IL-6 levels, *Parabacteroides* abundance and both IL-10 and IL-6 levels, *UBA1819* abundance and IL-4 levels, and *Clostridia* abundance and zonulin levels. In contrast, positive correlations were identified between *Alistipes* abundance and IL-10 levels, *Acidaminococcus* abundance and both IFN-γ and TNF-α levels, *Dialister* abundance and both TNF-α and IL-2 levels, and *Clostridia*

abundance and both IL-17 and IL-10 levels. Similarly, when analyzing other timepoints, we identified significant correlations between intestinal microbiota features, cytokine levels and zonulin (see **Figure 2A-2F**).

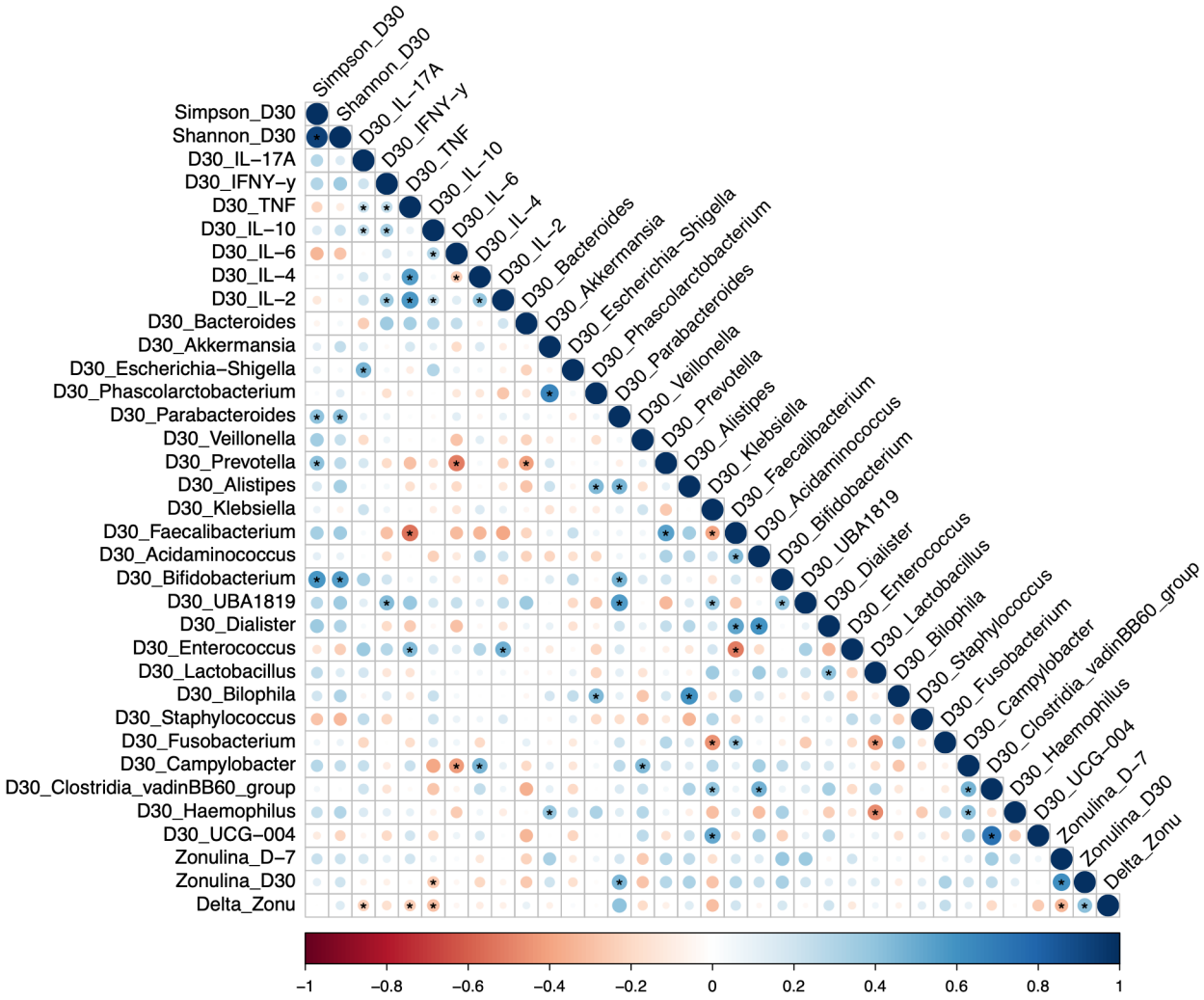
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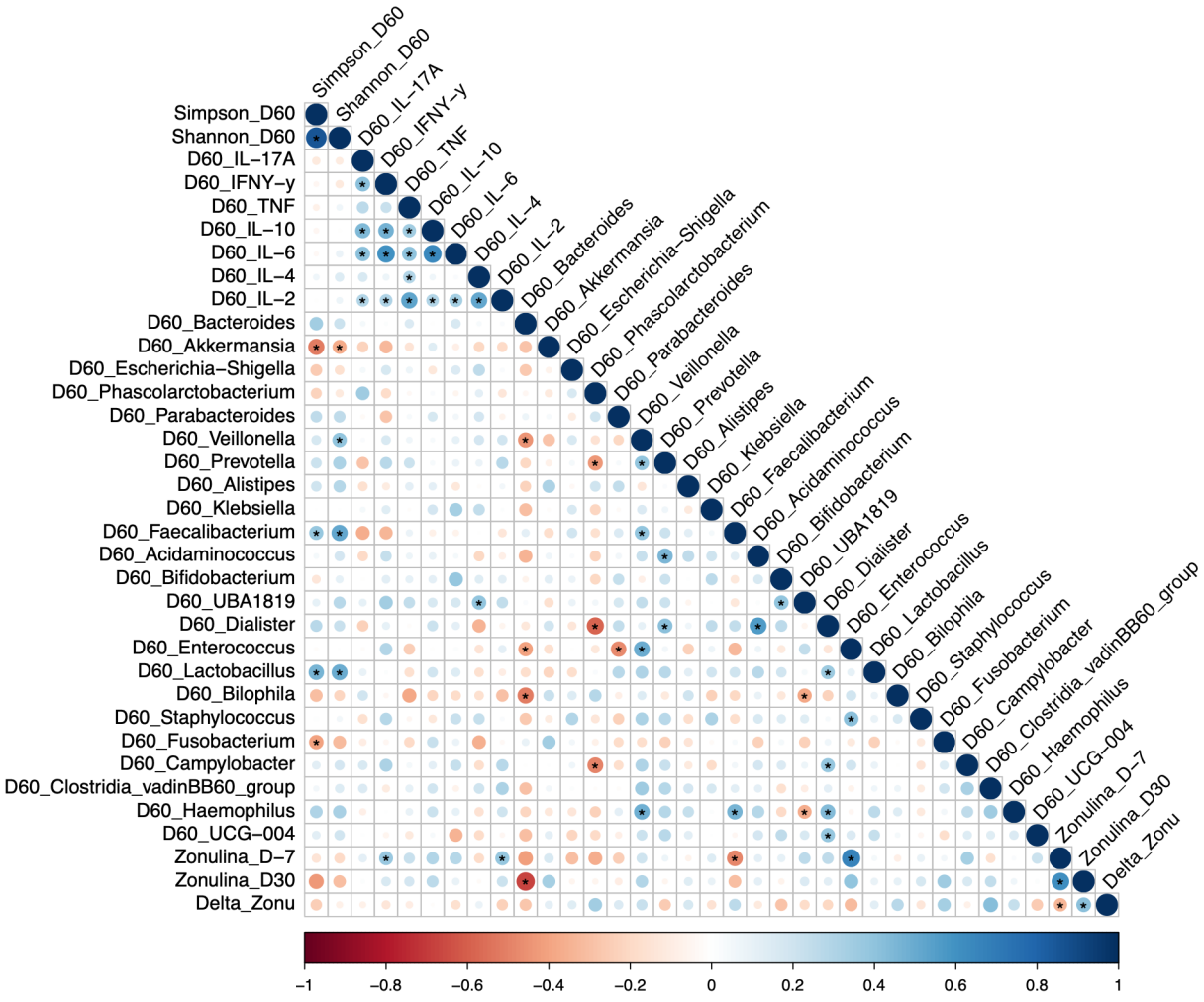
B


Simpson_D0
Shannon_D0
D0_IL−17A
D0_IFNY−y
D0_TNF
D0_IL−10
D0_IL−6
D0_IL−4
D0_IL−2
D0_Bacteroides
D0_Akkermansia
D0_Escherichia−Shigella
D0_Phascolarctobacterium
D0_Parabacteroides
D0_Veillonella
D0_Prevotella
D0_Alistipes
D0_Klebsiella
D0_Faecalibacterium
D0_Acidaminococcus
D0_Bifidobacterium
D0_UBA1819
D0_Dialister
D0_Enterococcus
D0_Lactobacillus
D0_Bilophila
D0_Staphylococcus
D0_Fusobacterium
D0_Campylobacter
D0_Clostridia_vadinBB60_group
D0_Haemophilus
D0_UCG−004
Zonulina_D−7
Zonulina_D30
Delta_Zonu
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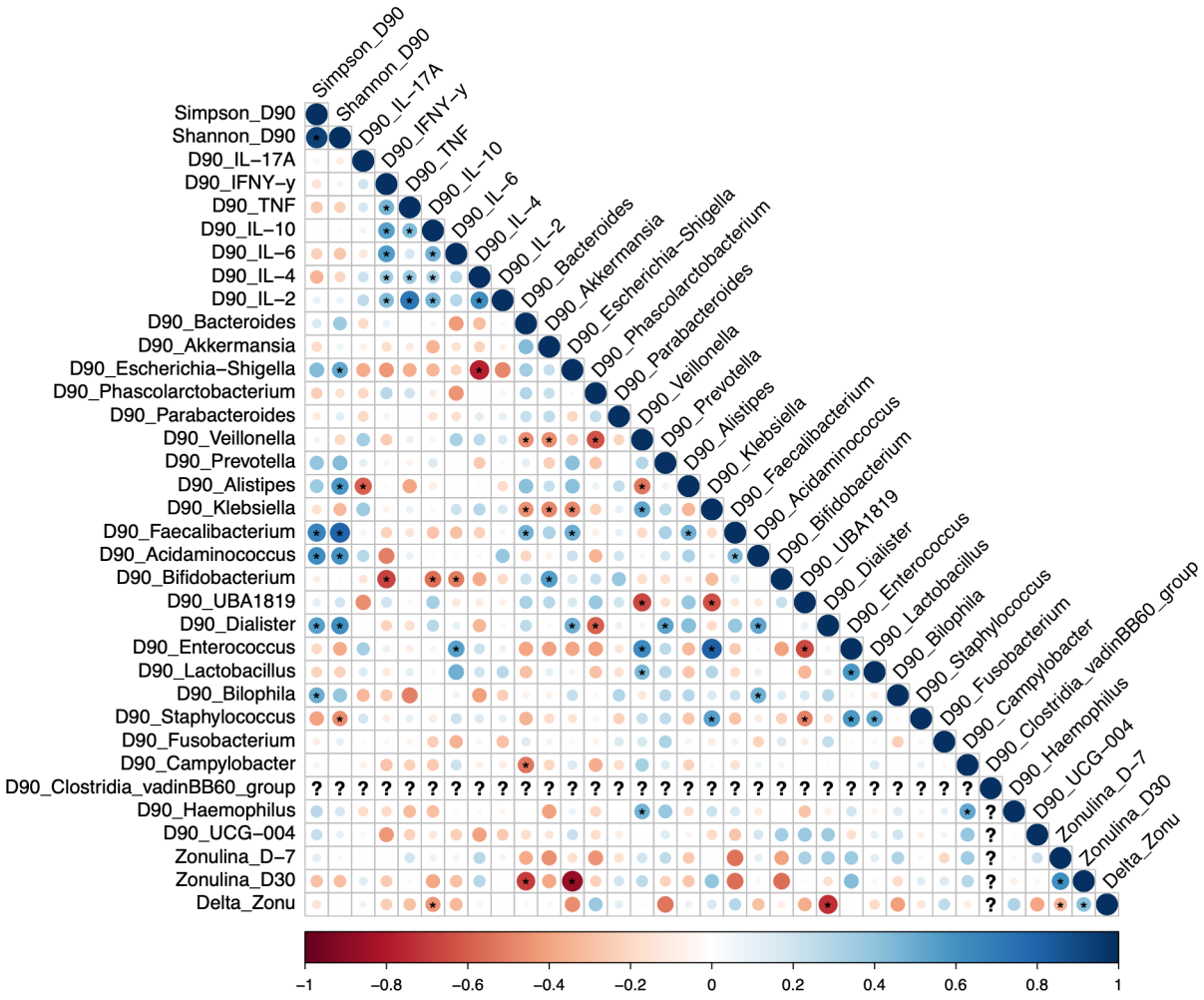
C


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D30_IFNY−y
D30_TNF
D30_IL−10
D30_IL−6
D30_IL−4
D30_IL−2
D30_Bacteroides
D30_Akkermansia
D30_Escherichia−Shigella
D30_Phascolarctobacterium
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D30_Campylobacter
D30_Clostridia_vadinBB60_group
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D30_UCG−004
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D


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D60_IFNY−y
D60_TNF
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D60_IL−6
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E


Simpson_D90
Shannon_D90
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D90_IFNY−y
D90_TNF
D90_IL−10
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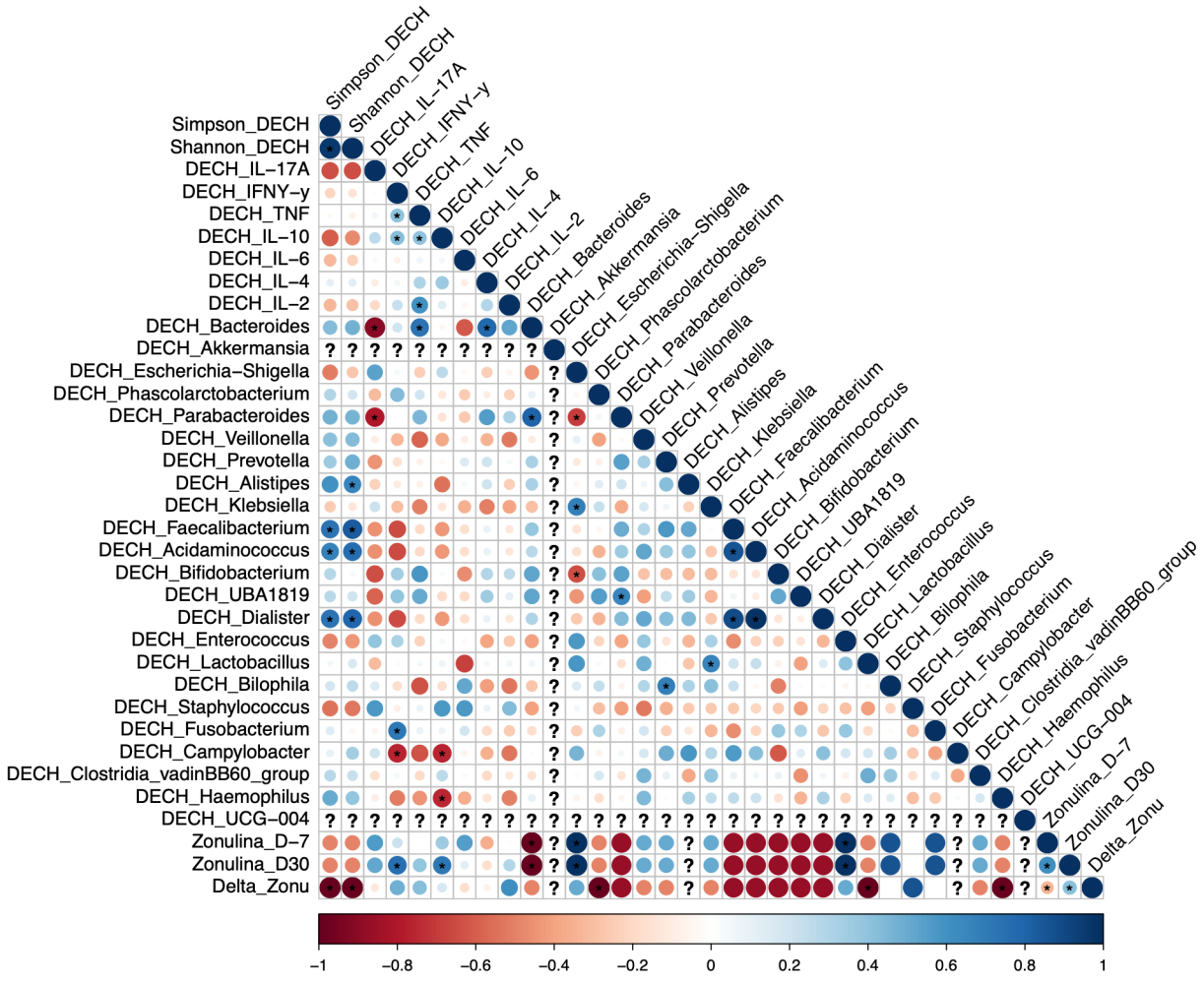


Figure 2. Heatmaps demonstrating correlation among intestinal microbiota features, zonulin and cytokines. (A) Samples from D-7. (B) Samples from D0. (C) Samples from D+30. (D) Samples from D+60. (E) Samples from D+90. (F) Samples from aGvHD. D = day; DECH = acute grave versus host disease; IL = interleukin; TNF = tumor necrosis factor; IFN = interferon; * = statistically significant correlation.

DISCUSSION

In this multicenter prospective study evaluating the dynamics and correlations among the intestinal microbiota, cytokines, and zonulin in patients undergoing allo-HSCT, we identified several key findings. First, compared with healthy controls, patients exhibited significantly lower alpha diversity and significantly higher serum zonulin levels. Second, although cytokine levels changed significantly throughout the allo-HSCT course, intestinal microbial diversity and

zonulin levels remained relatively stable over time. Finally, our heatmaps identified that several of these changes were significantly correlated with one another.

An important finding of this study is that intestinal microbiota and permeability showed significant alterations compared with healthy controls at multiple time points throughout the allo-HSCT course, suggesting that the crosstalk between them is substantially altered in the allo-HSCT setting. This is consistent with prior studies.[5,6,8,12,13] In a prior study including 606 patients undergoing allo-HSCT, intestinal microbial diversity was significantly lower compared with both healthy adult volunteers ($p < 0.001$) and the 212 participants from the Human Microbiome Project ($p < 0.001$).[5] In another study including 95 allo-HSCT patients, significantly increased intestinal permeability was identified compared with healthy controls based on three intestinal permeability markers: citrulline ($p < 0.001$), Reg3α ($p = 0.006$), and I-FABP ($p = 0.03$).[8] Interestingly, some of these studies demonstrated that these alterations are already present before the initiation of the conditioning regimen, suggesting that patients may begin allo-HSCT with an impaired intestinal barrier and altered microbiota.[5,8] Indeed, our findings demonstrated that zonulin levels were significantly elevated prior to allo-HSCT. However, we did not identify significant differences in overall intestinal microbiota diversity before transplantation. It is important to note that diversity indices capture both community richness and evenness and summarize these characteristics into a single metric. Therefore, similar diversity values do not necessarily indicate comparable microbial compositions, as differences in the relative abundance of specific taxa may still be present. Future studies should aim not only to further elucidate the crosstalk among these factors but also to evaluate additional metrics of intestinal microbiota composition and function before allo-HSCT initiation.

Another important finding of this study was the significant longitudinal changes observed in cytokine levels throughout the allo-HSCT course. Interestingly, zonulin levels did not show significant variation over time, and potential explanations for this finding have been extensively discussed in our previous study.[4] Regarding intestinal microbiota diversity throughout the allo-HSCT course, we identified only two statistically significant differences, with higher diversity values observed at D−7 compared with D+30 and D+60. This is partially supported by previous studies that have demonstrated significant temporal changes in both intestinal microbial diversity and cytokine profiles throughout the allo-HSCT course.[14,15] For example, in a previous study of 30 allo-HSCT recipients, IL-6, IL-10, and IFN-γ reached their peak levels on D+7, +21, and +28, respectively.[14] Similarly, in another study including 52 allo-HSCT recipients, IL-6, TNF-α, IL-8, and IL-10 peaked on D+21, +21, +7, and +28, respectively.[15] It is interesting to note that, although our data demonstrated significant cytokine dynamics, our cohort exhibited a distinct kinetic profile. For example, our findings identified earlier peaks for IL-6 and IL-10, occurring around D0, and a later peak for TNF-α, occurring around D+180. These differences in cytokine kinetics may be due to distinct patterns of intestinal microbiota dynamics. Although our findings did not identify significant changes in overall intestinal microbiota diversity throughout the allo-HSCT course, previous studies have consistently reported a decline in microbial diversity following allo-HSCT, with levels that may remain below baseline even months after transplantation.[7,12,16,17] This distinct microbiota trajectory observed in our cohort may be attributed to several factors, including differences in antibiotic exposure, dietary patterns, and potentially a more resilient baseline microbial community.[10,12,16,17] Other potential factors contributing to these distinct patterns may include differences in sampling time points, and

analytical methodologies.[14,15] Therefore, future studies should investigate the factors underlying these distinct kinetic patterns and determine their potential impact on clinical outcomes.

Our final major finding was the significant correlations observed among intestinal microbiota features, cytokines, and zonulin levels. Indeed, growing evidence has highlighted the complex crosstalk among these components, supporting the concept of the gut barrier–immune axis in the context of allo-HSCT.[1,14,15,18–20] Initially, evidence from preclinical studies has demonstrated that the intestinal microbiota may influence immune reconstitution after allo-HSCT through NOD1-dependent signaling mediated by microbe-derived ligands.[18] Subsequently, clinical studies provided further evidence supporting this concept in the allo-HSCT setting. For example, in a study including 894 allo-HSCT recipients, with 2,067 stool samples and 2,370 blood samples analyzed, higher intestinal alpha diversity was independently associated with higher CD4+ T-cell counts at 3 months after allo-HSCT ($p < 0.001$).[18] Additionally, increased relative abundance of *Staphylococcus* was associated with impaired CD4+ T-cell recovery during early phases after allo-HSCT.[18] Similarly, in another study including 1,582 allo-HSCT recipients, higher abundances of *Staphylococcus* and *Ruminococcus* were associated with higher lymphocyte counts during the post-transplant period.[18,20]

In our study, we specifically analyzed selected SCFA-producing genera based on prior evidence suggesting their role in regulating both immune responses and intestinal barrier integrity. Although the mechanisms underlying these interactions were not explored in this study, they may include histone acetylation, activation of G protein-coupled receptors, and modulation of NLRP3 inflammasome activity.[21,22] It is worth noting that our findings demonstrated several significant correlations between these genera and cytokine levels. For instance, we identified a significant negative correlation between *Bifidobacterium* abundance and IL-6 and IFN-γ levels in

samples collected at D+90. In the literature, *Bifidobacterium* is a well recognize genus that is used as probiotic to protect against inflammatory bowel disease due to their actions that improve both gut barrier integrity and host immune modulation.[23–26] It has been shown that specific strains of this genus can restore the production of thigh junction proteins such as occludin, ZO-1 and claudin due to a decrease production of both TNF and IL-6.[23–26] Another interesting finding from our study was the significant negative correlation between *Clostridia* abundance and zonulin levels. Members of the class Clostridia are important commensal bacteria that contribute to intestinal homeostasis through the production of SCFAs, especially butyrate.[27] Butyrate has been shown to promote intestinal barrier integrity by enhancing epithelial tight junction assembly, regulating inflammatory responses, and inducing the expansion of colonic regulatory T cells (Tregs).[28,29] Together with our findings, these studies highlight the potential role of the gut barrier–immune axis in the allo-HSCT setting. Nevertheless, it is important to acknowledge that our analysis was restricted to the genus level, and previous studies have demonstrated that these associations may be highly dependent on specific bacterial species and strains. Therefore, future studies should incorporate methodologies that enable more detailed characterization at the species level to provide a more precise understanding of these microbial–immune interactions. Furthermore, future investigations should not only characterize these associations but also evaluate their prognostic value following allo-HSCT.

The primary strength of this study is its comprehensive evaluation of the gut barrier–immune axis in the allo-HSCT setting. Nevertheless, several limitations should be acknowledged. First, our assessment of the immune component was limited to cytokine measurements, and we did not evaluate immune reconstitution after allo-HSCT. Characterizing immune cell reconstitution would have provided a more comprehensive understanding of the

crosstalk among the intestinal microbiota, intestinal permeability, and the immune system. Second, our assessment of intestinal permeability was restricted to zonulin measurements. Although zonulin has been validated as a marker of intestinal permeability in other clinical settings, its validity in the allo-HSCT context remains uncertain.[4] Furthermore, zonulin primarily reflects paracellular permeability, whereas extensive epithelial injury following allo-HSCT may also increase transcellular permeability. Finally, another limitation of our study is the absence of information regarding CD4+-selected grafts in our cohort. Because immune reconstitution and cytokine profiles may differ according to graft manipulation strategies, differences between CD4+-selected and conventional allo-HSCT may have influenced our findings.[18] Despite these limitations, this study provides novel insights into the dynamics and crosstalk of the gut barrier-immune axis in the allo-HSCT setting. Future studies should adopt a broader approach by assessing multiple markers of intestinal permeability and immune reconstitution to provide a more comprehensive characterization of the gut barrier–immune axis in the allo-HSCT setting.

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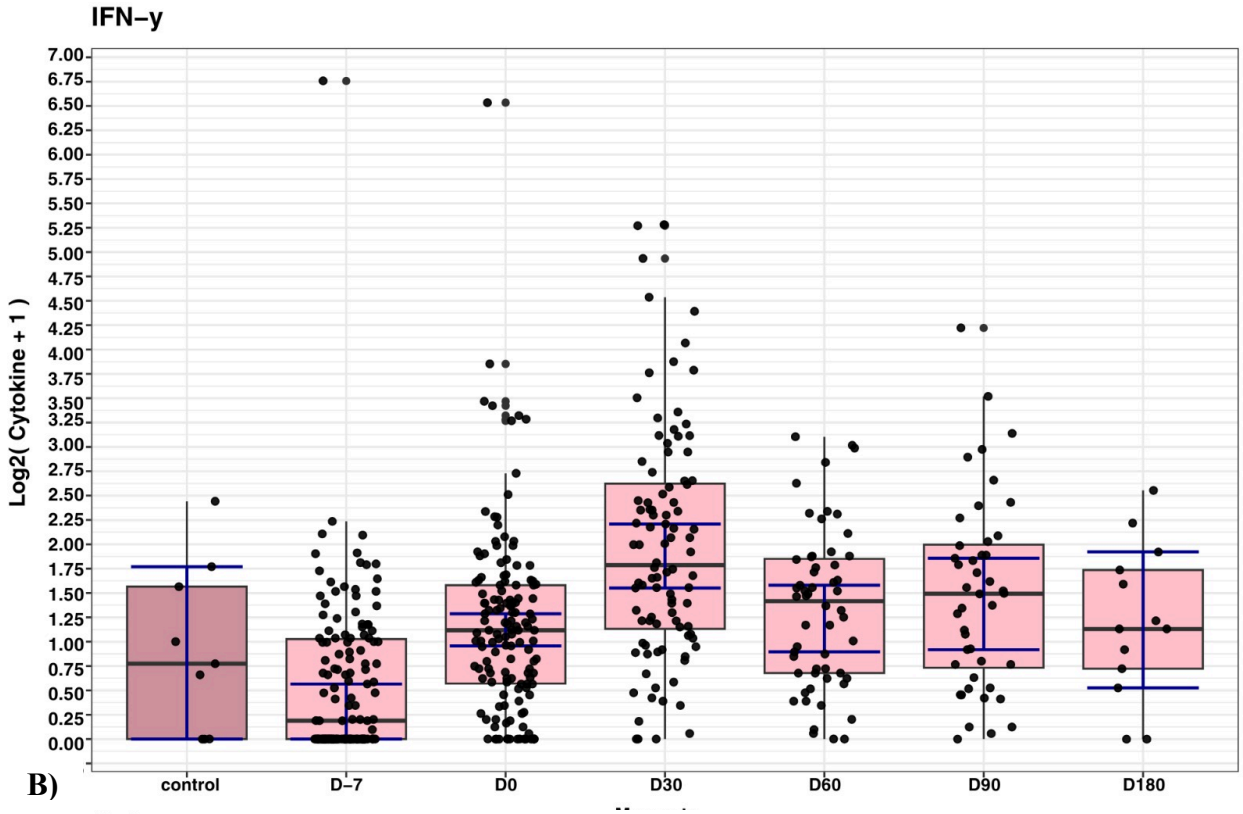
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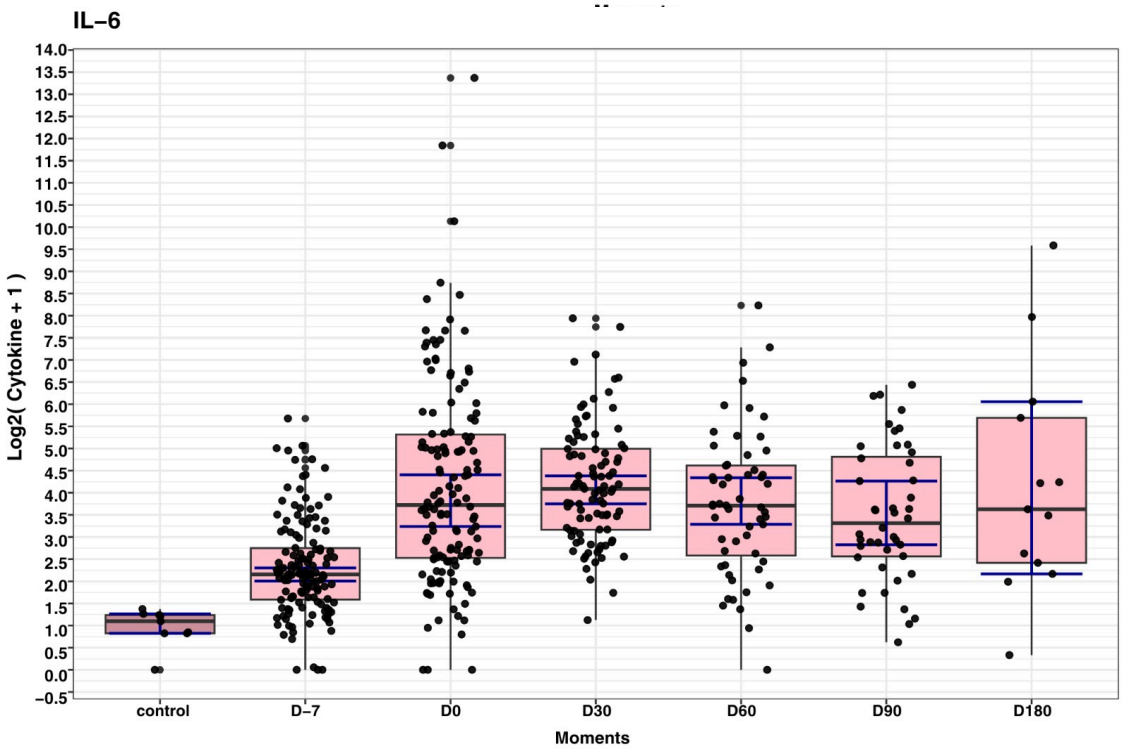
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Supplementary Figures

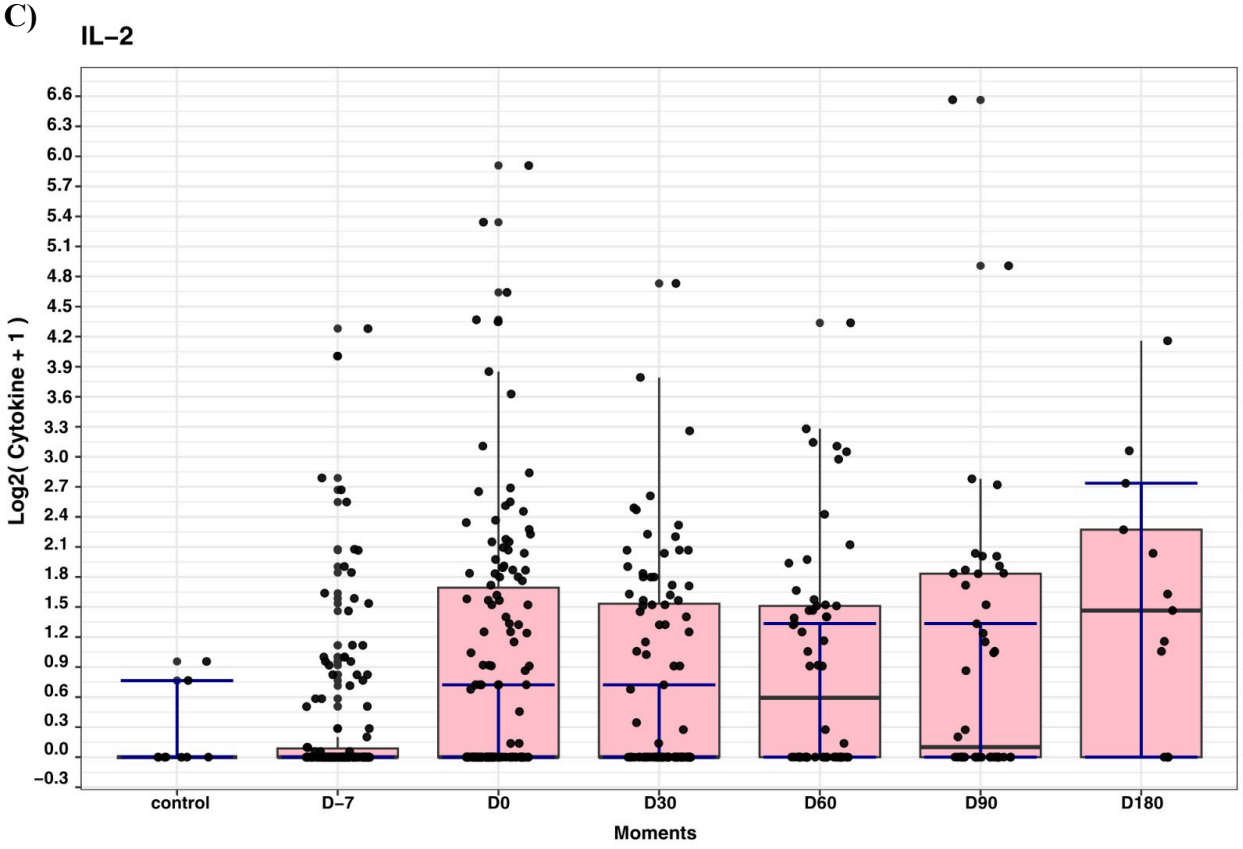
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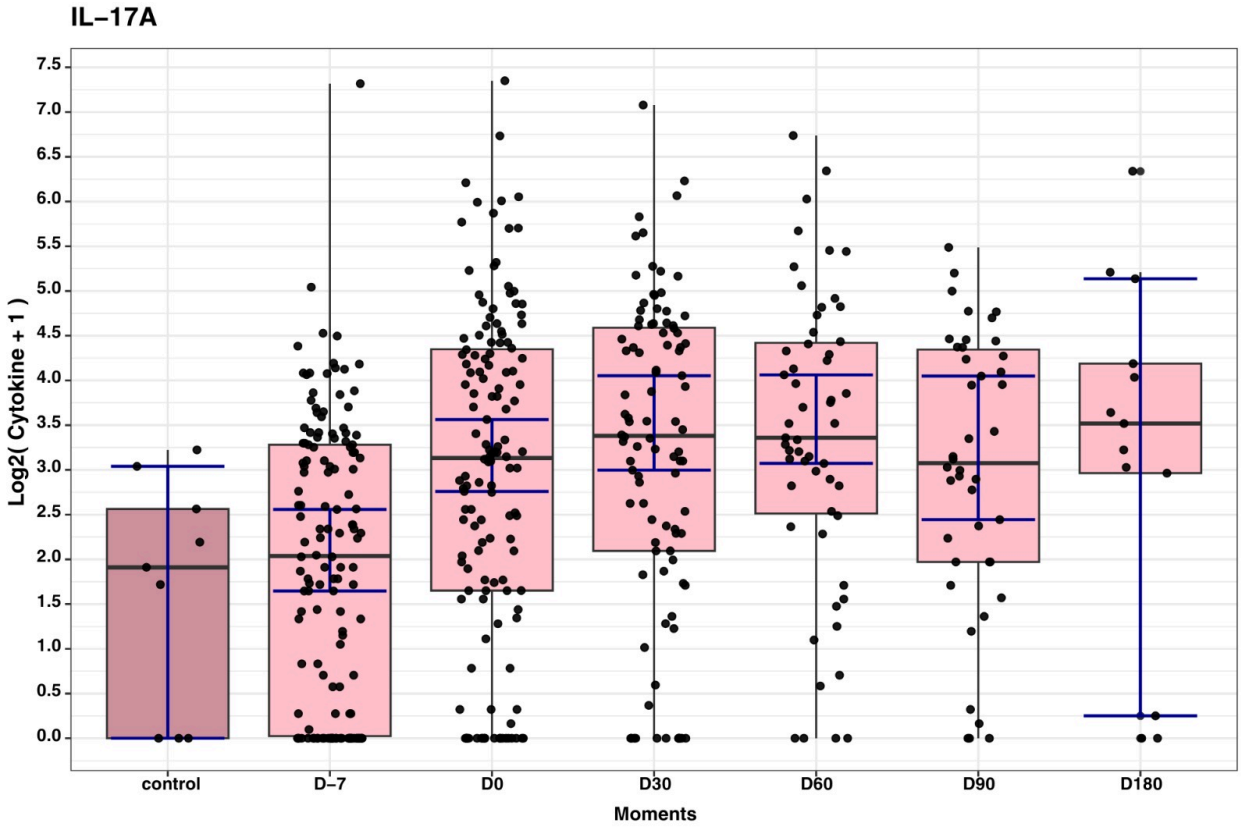
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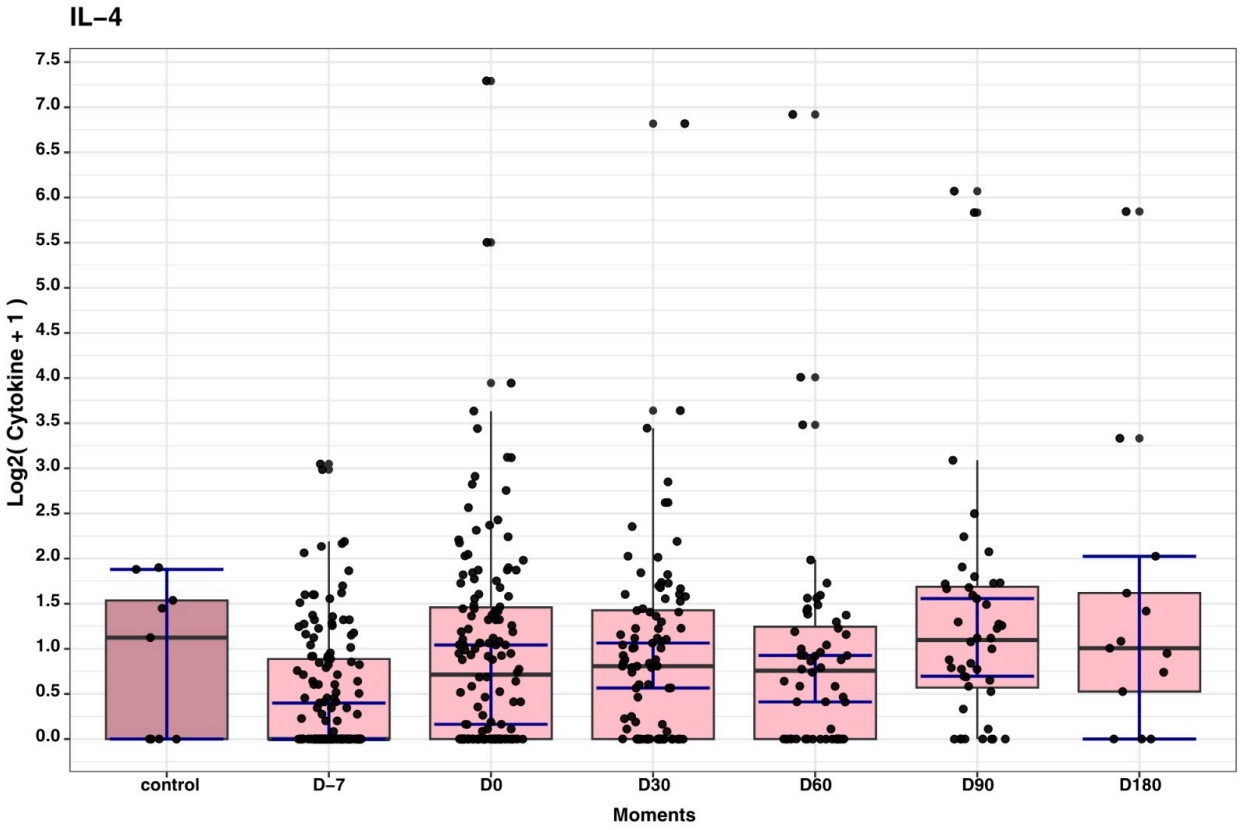
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2.7
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0.0
−0.3
control
D−7
D0
D30
D60
D90
D180
Moments

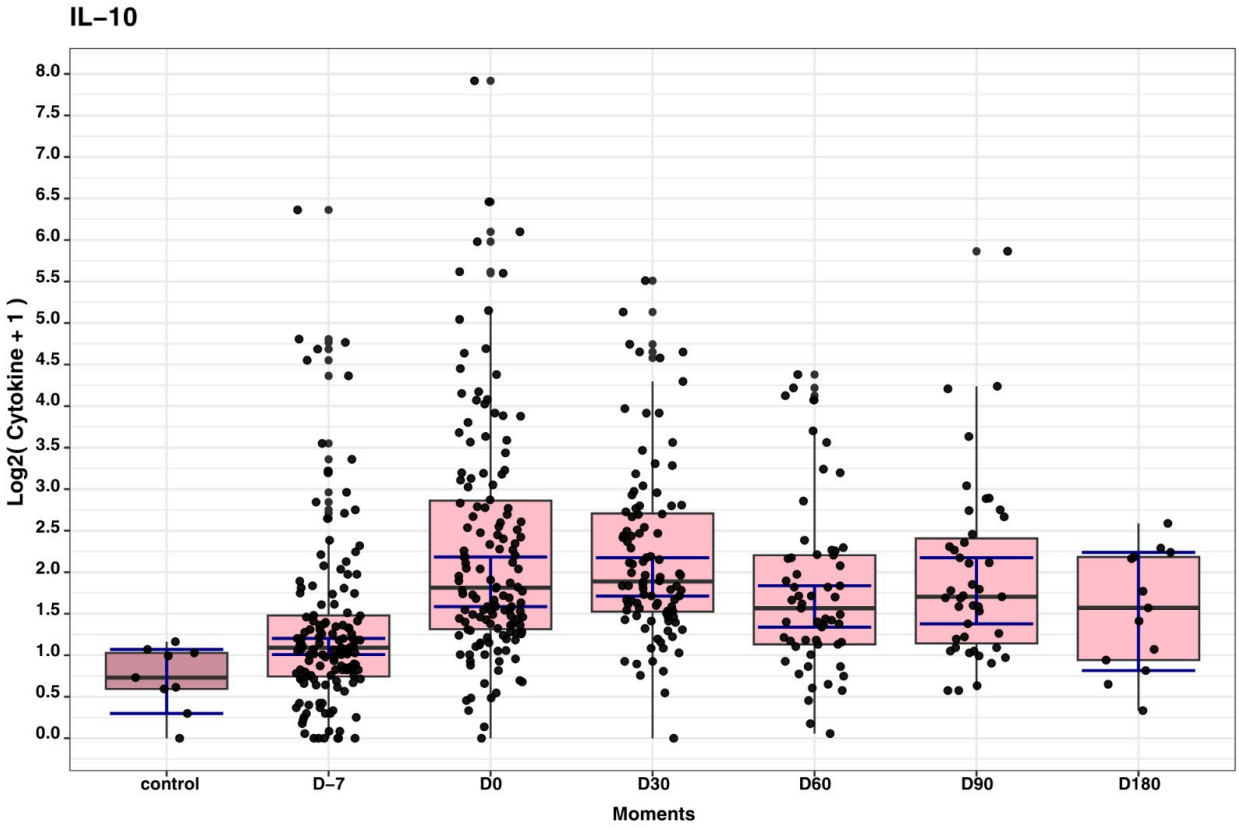
D)


IL−17A
Log2(Cytokine + 1)
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
control
D−7
D0
D30
D60
D90
D180
Moments

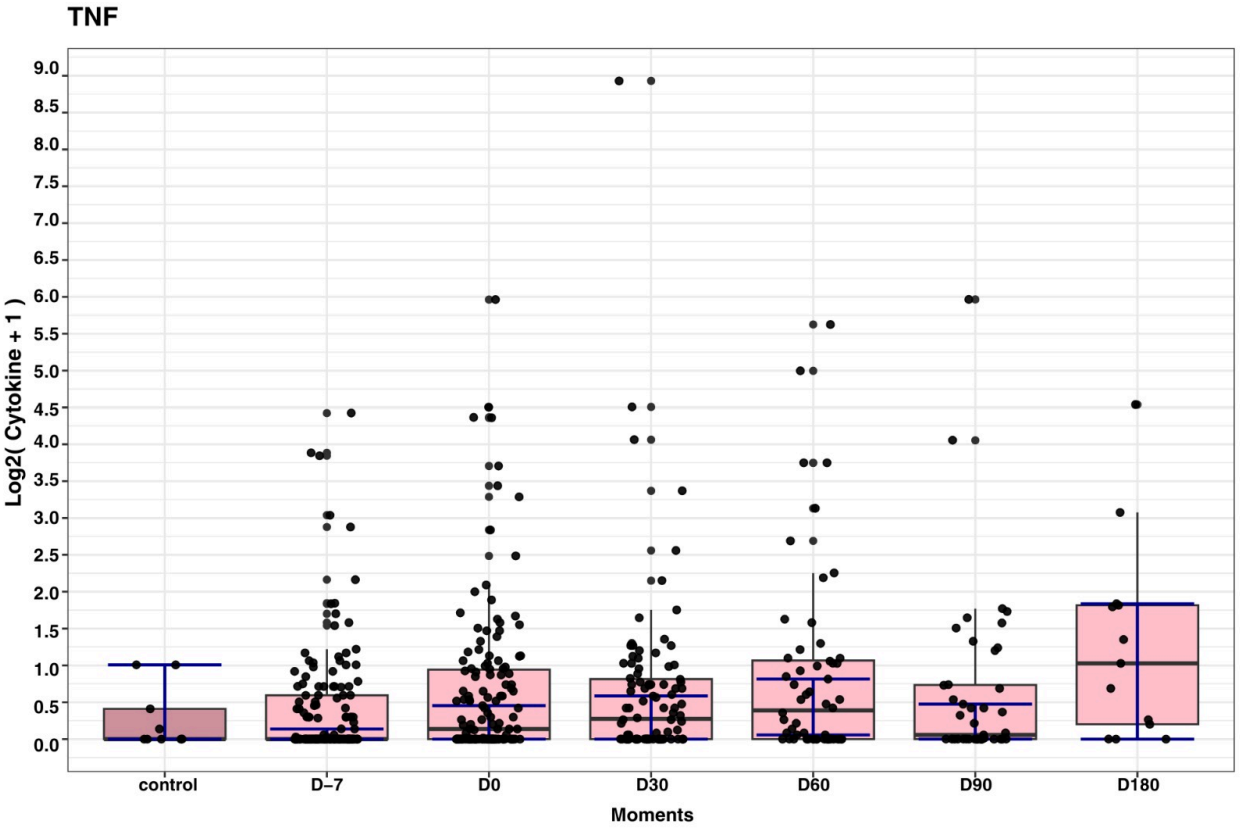
E)


IL−4
Log2(Cytokine + 1)
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
control
D−7
D0
D30
D60
D90
D180
Moments

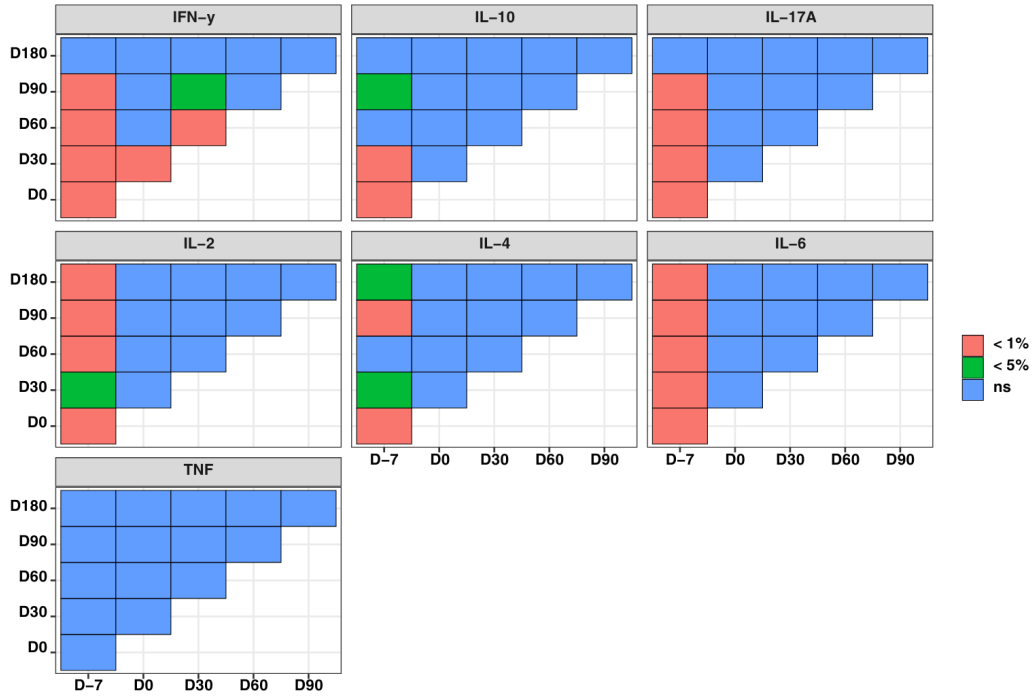
F)


IL−10
Log2(Cytokine + 1)
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
control
D−7
D0
D30
D60
D90
D180
Moments

G)



Supplementary Figure 1. Temporal dynamics of cytokines during the allo-HSCT course compared with healthy controls. Boxplots illustrate cytokine levels across different allo-HSCT time points and in healthy controls for (A) IFN-γ, (B) IL-6, (C) IL-2, (D) IL-17A, (E) IL-4, (F) IL-10, and (G) TNF. D, day; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon.



Supplementary Figure 2. P-values from pairwise comparisons used to evaluate cytokine dynamics across allo-HSCT time points, as presented in Supplementary Figure 1. D = day; NS = non-significant; IL = interleukin; TNF = tumor necrosis factor; IFN = interferon.

Conclusão Final

Em conclusão, nosso estudo caracterizou de forma integrada a microbiota intestinal, a permeabilidade intestinal e o perfil de citocinas em uma coorte de pacientes brasileiros submetidos ao alo-TMO. A análise conjunta desses parâmetros permitiu identificar características particulares, ou *fingerprints*, dessa população, contribuindo para uma compreensão mais abrangente das complexas interações entre a microbiota intestinal, a integridade da barreira intestinal e a resposta imunológica no contexto do alo-TMO. Esses achados ampliam o conhecimento sobre essa interação em uma população brasileira e fornecem uma base para futuras investigações sobre seu potencial impacto nos desfechos pós-transplante.

Em relação à microbiota intestinal, identificamos um *fingerprint* aparentemente característico da população brasileira, marcado pela baixa frequência de eventos de dominância por *Enterococcus*. Embora eventos de dominância tenham sido observados com frequência ao longo do alo-TMO, os gêneros bacterianos envolvidos nesses eventos diferiram substancialmente daqueles descritos em coortes internacionais, sugerindo que a microbiota durante o alo-TMO pode apresentar importantes variações geográficas e populacionais. Esse achado reforça a necessidade de considerar características regionais da microbiota na interpretação de padrões de disbiose em pacientes submetidos ao alo-TMO. Em nossa coorte, não foram identificadas associações relevantes entre os eventos de dominância e os desfechos clínicos avaliados. Entretanto, esses resultados devem ser interpretados com cautela, uma vez que as análises de associação com desfechos foram realizadas em um número relativamente pequeno de pacientes, o que pode ter limitado o poder estatístico para detectar associações clinicamente relevantes. Dessa forma, a relevância prognóstica dos eventos de dominância identificados em nossa população permanece ainda desconhecida e deverá ser investigada em coortes maiores.

A avaliação longitudinal da permeabilidade intestinal também revelou um *fingerprint* distinto em nossa população. Observamos níveis elevados de zonulina já no momento de entrada dos pacientes no alo-TMO, com manutenção desses níveis ao longo do período pós-transplante e ausência de uma dinâmica longitudinal significativa. Esse padrão sugere que, em nossa população, a alteração da permeabilidade intestinal mediada por zonulina pode estar estabelecida antes mesmo da realização do alo-TMO, diferindo de uma interpretação na qual o aumento da permeabilidade seria predominantemente uma consequência das exposições associadas ao

transplante. Além disso, a ausência de alterações significativas ao longo do período avaliado pode indicar que a zonulina isoladamente apresenta potencial limitado como marcador prognóstico durante o alo-TMO. Esses achados também levantam a possibilidade de que outros mecanismos envolvidos na regulação da permeabilidade intestinal, incluindo alterações estruturais da barreira epitelial, possam desempenhar papel mais relevante nesse contexto e devem ser investigados em estudos futuros.

A análise integrada das relações entre microbiota intestinal, permeabilidade e citocinas evidenciou associações significativas entre esses componentes, reforçando a importância do *cross-talk* entre microbiota–barreira intestinal–imunidade no contexto do alo-TMO. Foram identificadas correlações significativas envolvendo diferentes gêneros bacterianos produtores de ácidos graxos de cadeia curta (SCFAs), marcadores de permeabilidade intestinal e citocinas. Entre os achados de maior relevância, observamos uma correlação negativa entre a abundância de *Bifidobacterium* e os níveis de IL-6 e IFN-γ, além de uma correlação negativa entre a abundância de *Clostridia* e os níveis séricos de zonulina. Esses resultados são particularmente relevantes considerando que membros desses grupos bacterianos estão descritos na literatura como importantes componentes da homeostase intestinal, especialmente por meio da produção de SCFAs e de potenciais efeitos sobre a integridade da barreira epitelial e a modulação da resposta imune. Dessa forma, nossos achados reforçam a hipótese de que alterações na composição da microbiota possam estar funcionalmente relacionadas tanto à integridade da barreira intestinal quanto à produção de citocinas ao longo do alo-TMO. Embora essas associações contribuam para uma compreensão mais integrada do eixo microbiota–barreira intestinal–imunidade, é importante destacar que as análises realizadas neste estudo foram conduzidas predominantemente em nível de gênero. Evidências prévias indicam que os efeitos sobre a barreira intestinal e a resposta imune podem ser altamente dependentes da espécie. Portanto, a caracterização em maior resolução taxonômica será fundamental para determinar quais microrganismos específicos estão envolvidos nessas interações e para compreender melhor sua relevância biológica e clínica.

De forma integrada, os resultados deste projeto demonstram que a microbiota intestinal, a permeabilidade intestinal e os níveis de citocinas constituem componentes interdependentes no contexto do alo-TMO e que sua caracterização em uma população brasileira revela

particularidades que podem não ser completamente capturadas por estudos conduzidos em outras populações. A identificação de *fingerprints* distintos, associada às correlações observadas entre gêneros bacterianos, zonulina e citocinas, contribui para ampliar a compreensão da complexa interação entre o hospedeiro e sua microbiota durante o transplante. Ao mesmo tempo, os resultados ressaltam que a relevância prognóstica desses marcadores ainda precisa ser estabelecida de maneira mais robusta. Portanto, estudos futuros, com maior tamanho amostral, deverão empregar abordagens estatísticas mais robustas e modelos multivariados capazes de integrar simultaneamente dados de microbiota, marcadores inflamatórios, permeabilidade intestinal e características sociodemográficas e clínicas, possibilitando a construção de escores preditivos e uma avaliação mais precisa do risco de complicações e desfechos após o alo-TMO.

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