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GUT MICROBIOTA EFFECTS IN HEMATOPOIETIC STEM CELL TRANSPLANT PATIENTS

ALLOJENİK KÖK HÜCRE NAKİLLERİNDE MİKROBİYOTA ETKİSİ

Ekin Ece GÜRER[1], Fatma SAVRAN OĞUZ[1], Sevgi BEŞIŞIK KALAYOĞLU[2], Zerrin AKTAŞ[3], Zafer GÜLBAŞ[4], Mustafa Oral ÖNCÜL[5], Uğur SEZERMAN[6]

[1]Istanbul University, Istanbul Faculty of Medicine, Department of Medical Biology, Istanbul, Turkiye
[2]Istanbul University, Istanbul Faculty of Medicine, Department of Internal Medicine, Hematology Division, Therapeutic Apheresis Unit, Istanbul, Turkiye
[3]Istanbul University, Istanbul Faculty of Medicine, Department of Medical Microbiyology, Istanbul, Turkiye
[4]John Hopkins Anadolu Health Center, Department of Hematologic Oncology, Kocaeli, Turkiye
[5]Istanbul University, Istanbul Faculty of Medicine, Department of Enfection Dissease and Clinical Microbiyology, Istanbul, Turkiye
[6]Acıbadem University, Faculty of Medicine, Department of Biostatistics and Medical Informatics, Istanbul, Turkiye

ORCID IDs of the authors: E.E.G. 0000-0002-1758-9814; F.S.O. 0000-0002-6018-8936; S.B.K. 0000-0002-9310-1278; Z.A. 0000-0002-5998-0440; Z.G. 0000-0002-4709-0627; M.O.Ö. 0000-0002-1681-1866, U.S. 0000-0003-0905-6783

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ABSTRACT

Objective: In our study, we analyzed gut microbiota in allo-HSCT patients and aimed to evaluate the relationship of gut microbiota with transplant complications, mainly GVHD.

Materials and Methods: A total of 25 adult recipients and donors who underwent allo-HSCT at Istanbul Anadolu Medical Center were included in the study. Stool samples were collected twice, before chemotherapy regimen and after allo-HSCT. Samples were analyzed by High Melting (HRM) Analysis and Next Generation Sequencing (NGS) methods after nucleic acid isolation. Sequencing was done with Illumina MiSeq. Bacteria Silva database was used for taxonomic classification and QIIME 2 programs were used for analysis. Statistical analyses were carried out with the R statistical programming language.

Results: Twenty-five allo-HKHN recipients were included in the study. The mean age was 46.24±14.86 years in recipients and 43.40±13.20 years in donors. Gender distribution was M/F: 15/10 in patients and M/F: 17/8 in donors. Recipient and donor sibling HLA match was 10/10. The rate of GVHD associated with Allo-HSCT was 16%, and the relapse rate was 16%. It was observed that the Firmicutes and Proteobacteria phyla changed significantly before and after transplantation. The number of Entereccocus species was found to be higher in patients who developed GVHD and died. The loss of diversity was found to be statistically significant in the pre-transplant and post-engraftment samples of the patients.

ÖZET

Amaç: Çalışmamızda allo-hematopoetik kök hücre nakli (allo-HKHN) uygulanmış hastaların mikrobiyota analizleri yapılmıştır. Nakile ve tedavilere bağlı olarak değişen mikrobiyota florasının engrafman ve Graft-Versus-Host Hastalığı (GVHH) gelişimi ile ilişkisinin gösterilmesi amaçlanmıştır.

Gereç ve Yöntem: İstanbul Anadolu Sağlık Merkezi'nde allo-HKHN uygulanan toplam 25 yetişkin alıcı ve vericileri çalışmaya dahil edildi. Dışkı örnekleri, Hazırlık Rejimi (HR) öncesi ve allo-HKHN sonrası toplamda 2 kez alınmıştır. Örnekler, nükleik asit izolasyonu yapıldıktan sonra, Çözünürlüklü Erime Analizi (HRM) ve Yeni Nesil Dizileme (YND) yöntemi ile analiz edilmiştir. Dizileme işlemi, Illumina MiSeq cihazı ile yapılmıştır. Taksonomik sınıflandırma için Bacteria Silva veri bankası ve analiz için QIIME 2 programları kullanılmıştır. İstatistiksel analizler ise R istatistiksel programlama dili ile gerçekleştirilmiştir.

Bulgular: Çalışmaya dahil edilen alıcılarda yaş ortalaması 46,24±14,86 (18-71) yıl, vericilerde 43,40±13,20 yıl (11-61) olarak saptandı. Hastalarda cinsiyet dağılımı; E/K: 15/10 vericilerde E/K: 17/8 idi. Alıcı ve verici kardeş HLA uyumu 10/10 idi. Allo-HKHN ile ilişkili GVHH oranı %16, relaps oranı ise %16 bulundu. Nakil öncesi ve sonrası Firmicutes ve Proteobacteria filumlarının önemli ölçüde değiştiği gözlendi. GVHH geliştiren ve ex olan hastalarda Entereccocus türlerinin sayısı daha fazla bulundu. Hastaların nakil öncesi ve engrafman sonrası örneklerinde çeşitlilik kaybının istatistiksel olarak anlamlı olduğu saptandı.

Corresponding author/İletişim kurulacak yazar: Ekin Ece GÜRER – ekinecegurer@ogr.iu.edu.tr

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Conclusion: Gut microbiota diversity may guide the monitoring of GVHD and also may be manipulated for the treatment of GVHD. It is thought that increasing the diversity of commensal bacteria can also positively affect the prognosis of the disease.

Keywords: intestinal microbiota, blood diseases, hematopoietic stem cell transplantation, HLA

Sonuç: Allo-HKHN sonrası artan patojen bakteriler ile hastalığın şiddetlenmesi, intestinal flora izlemi ile GVHH koruma ve/veya tedavisinde yönlendirici olabileceğini göstermektedir. Kommensal bakterilerin çeşitliliğinin arttırılmasıyla beraber hastalığın prognozunu da olumlu yönde etkileyebileceğini düşündürmektedir.

Anahtar Kelimeler: Bağırsak mikrobiyotası, kan hastalıkları, hematopoetik kök hücre nakli, HLA

INTRODUCTION

The intestinal microbiota is symbiotic, commensal, and pathogenic microorganisms located on the mucosal surface and in certain niches (1). It contributes to food digestion and maintains the structural integrity of intestinal mucosa, thus preventing the colonization of pathogens. It takes part in the production of ATP with the short-chain fatty acids (SCFA), the production of vitamin B and vitamin K, and inregulation of the cells which are involved in immune recognition and modulation (2).

Recently, intestinal microbiota have become a different target for investigating the pathogenesis and clinical outcomes of disorders. Intestinal microbiota studies regarding allogeneic hematopoietic stem cell transplantation (HSCT) patients are also increasing. Studies have shown associations between high diversity of the intestinal microbiota and a lower risk of transplant-related mortality (3).

Acute graft-versus-host disease (aGVHD) is a major complication of allogeneic HSCT and may be life threatening in some cases. aGVHD severity is graded clinically by the involvement of main target organs such as skin gastrointestinal tract and liver of host (4, 5).

Uric acid and ATP are secreted, and proinflammatory cytokines are released related with epithelial damage. The intestinal microbiota in this settingmay build up a new treatment issue by regulating homeostasis and the immune response (6).

In this study, we investigated intestinal microbiota in allogeneic HSCT patients. We aimed to reveal the microbiota change in the course of allo-HSCT and evaluated the relationship of microbiota with the main complications of allo-HSCT.

MATERIALS AND METHODS

A total of 25 adult allo-HSCT donors and patients who were hospitalized for HSCT in the Hematology and Transplantation Unit of Anadolu Medical Center Johns Hopkins hospital enrolled in the study. Stool samples were collected twice, just before the conditioning regimen and after 3 weeks of HSCT. The samples were stored at -80°C until collective studying.

Study approval was obtained from the Istanbul University, Istanbul Faculty of Medicine Clinical Research Ethics Committee, and all patients participated in the study after written informed consent was obtained (Date: 19.07.2019, No: 146386).

In the first stage, the nucleic acid isolation was performed from samples using the``nucleic acid isolation kit from feces``(Bioeksen, Turkiye). All samples were subjected to High-Resolution Melting Analysis (HRM) and New-Generation Sequencing (NGS) tests.

The microorganism DNA isolation procedure

The previously defined physical (bead fragmentation or sonication), chemical (SDS or CTAB), and biochemical (proteinase K, lysozyme) fragmentation methods for DNA isolation were applied in different combinations and the most effective methodwas determined. Silica columns were used for differentiating the DNA and protein molecules in fragmented cells, and RNA contamination was eliminated by the RNAase procedure. In the last stage of isolation, the DNA attached to the silica columns was dissolved in water with no DNase/Pyrogen and the nucleic acid concentration was determined with the help of a spectrophotometer. The 'OD260/OD280' ratio was adjusted to between '1.8-2.0,' 'OD260/OD230' ratio was adjusted to '2.0-2.2,' and the DNA studied in a concentration of at least 10 ng/ul (preferably 50-300 ng/µl).

High resolution melting (HRM) analysis

Amplification of 16S rDNA regions before HRM was performed by polymerase chain reaction (PCR). A standard DNA with a known sequence was included in all HRM analyses to obtain a reference HRM profile. The obtained temperature-fluorescence intensity raw data were analyzed using the Biospeedy® HRM Analysis Software (Bioeksen R&D Technologies Ltd. Şti., Turkiye) which is based on the statistical approach defined by Reja et al. (7). The profiles obtained as a result of HRM analysis were created using the Minitab 17 software program (Minitab, UK) and dendrograms were created by comparison with themselves. The calculation of the principal component analysis (PCA) ordinations and correlation analyses were performed using the Minitab 17 software program.

The groups identified as a result of HRM analysis were analyzed by combining the DNA of each group in themselves. An average of 460 bp, which included the V3-V4 regions of the 16S rRNA gene, was targeted. To obtain amplicon pools, an area of about 460 bp covering the V3-V4 region of the 16S rRNA gene was targeted (8).

Next-generation sequencing (NGS)

The groups identified after HRM analysis were analyzed by combining the DNA of each group in themselves. In the first PCR procedure, "Bio speedy® Proof Reading DNA Polymerase 2x Reaction Mix" was used and 200 nm was applied from all primers. The PCR process was performed on the Biorad CFX Connect Device.

The second PCR step of the purified PCR sample was performed using the Nextera XT Index Kit (Illumina, USA) of the Illumina sequencing adapters. MiSeq sequencing was performed after denaturation was enabled with temperature. 'Illumina MiSeq v3 reaction kits' were used in this process. 5% PhiX was included in all reactions for control purposes.

Statistical analysis

In microbiota analysis, the index and primary sequences were clipped, and then the original sequences were identified. The clipped original sequences were aligned using the SILVA rRNA database algorithm. Before this step, the SILVA database (taxonomy files and reference) (9) were cut, and it was ensured that there was only a V3-V4 region. Inappropriate sequences located at both ends of the arrays were removed using the filtering method, which allowed error checking. By doing pre-clustering, impurity was avoided. Chimera elimination was performed using the UCHIME (10) code. The classification was carried out by Mothur-Bayesian on arrays. The OTUs were grouped according to their phylotypes after the operational taxonomic unit (OTU) was selected and the taxonomic determination was made according to the SILVA rDNA database. The Bacteria Silva data bank was used for taxonomic classification, and QIIME 2 programs were used for analysis.

RESULTS

The demographic characteristics of the patients - donors are summarized in Table 1. The allo-HSCT protocol was the same in all patients. All transplant donors were related and, except for for one (cousin), all were siblings. All donors were HLA matched as 10/10.

Table 1: Demographic data of study groups

	Recipient (Mean±SD)	Donor (Mean±SD)
Age (year)	46.24±14.86	43.40±13.20
Gender (men/women)	15/10	17/8

Peripheral blood was the most frequently used (92%) stem cells source and all of the remaining were bone marrow.

HSCT indications are given in Table 2. Transplant related complications and aGVHD frequency are given in Table 3.

Table 2: Transplant indication disorders

	(%) n
HLA match	
HLA full match	100
HLA mismatch	0
Unrelated	0
Stem cell source	
Peripheral	92
Bone marrow	8
aGVHD prophylaxis	Cyclophosphamide, cyclosporine or tacrolimus, mycophenolate mofetil
Condition regimen	Busulfan and cyclophosphamide
	Total body irradiation (TBI) and cyclophosphamide
	Fludarabine+melphalan+ATG
	Treosulfan+fludarabine
Antimicrobial prophylaxis	Sulfamethoxazole+trimethoprim, fluconazole, acyclovir
Relaps	16
aGVHD	16
Full chimeric	20
Graft rejection	0

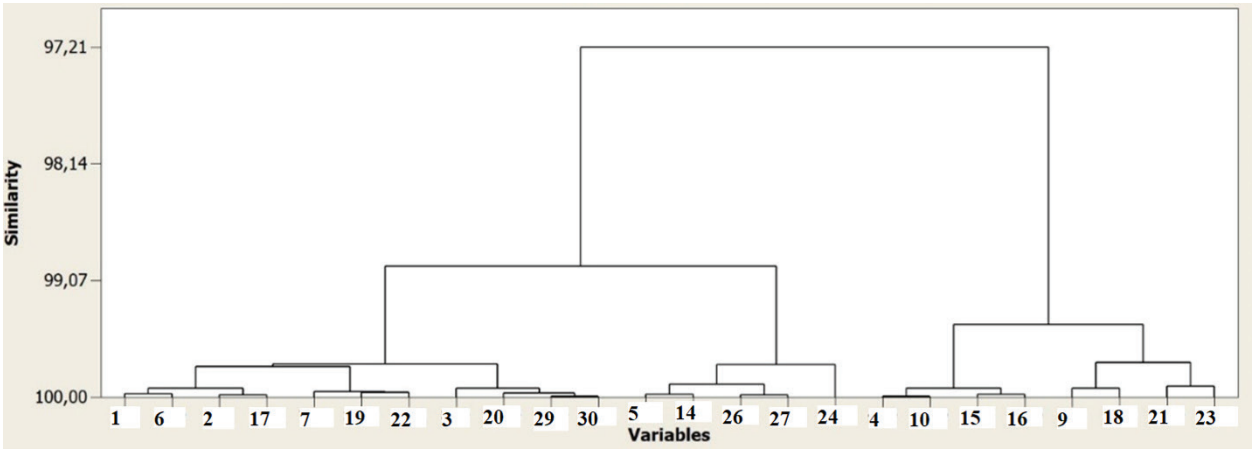
(HLA: human leukocyte antigen, aGVHD: acute graft-versus-host disease)

Table 3: Transplant complications and aGVHD frequency

Disease	**n=25**
AML	6
MDS	4
MM	2
NHL	3
HL	1
ALL	2
CML	2
CMML	1
IAA	1
PMF	1
CMPH	2
aGVHD	**n=4**
Grade I	3
Grade II	0
Grade III	0
Grade IV	1

AML: acute myeloid leukemia, MDS: myelodysplastic syndromes, MM: multiple myeloma, NHL: non-Hodgkin lymphoma, HL: Hodgkin lymphoma, ALL: acute lymphoblastic leukemia, CML: chronic myeloid leukemia, CMML: chronic myelomonocytic leukemia, IAA: idiopathic severe aplastic anemia, PMF: primary myelofibrosis, CMPH: chronic myeloproliferative disease, aGVHD: acute graft-versus-host disease

Figure 1 showed HRM analysis and dendrograms. Dendrogram results were grouped according to species' 100% similarity and pools were created. In total, three groups and 16 nucleic acid pools were created, including four for donorss' samples (Table 4), six for pre-transplant (Table 5), and six for post-transplant (Table 6) samples. All groups' samples were analyzed in detail using the NGS method.



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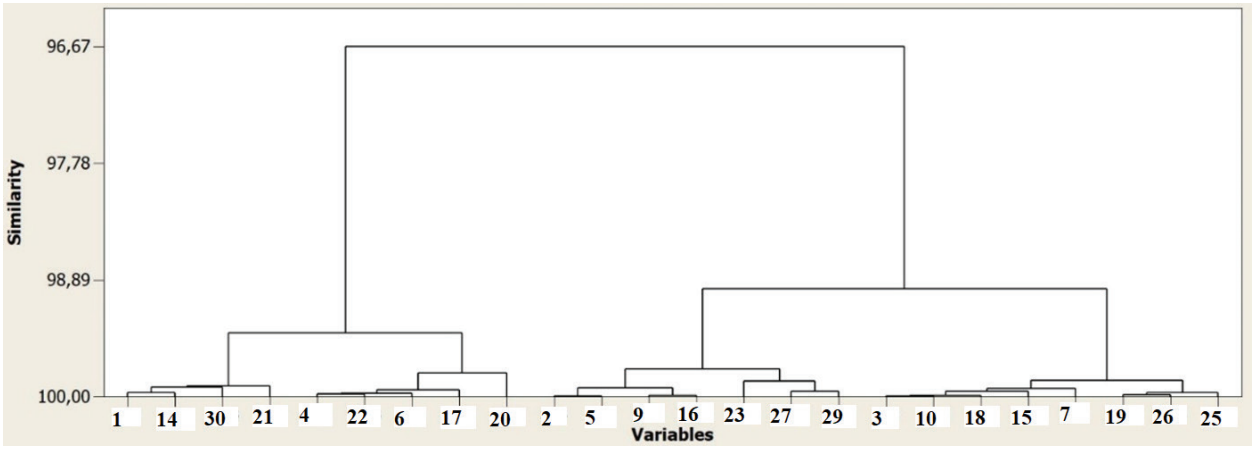

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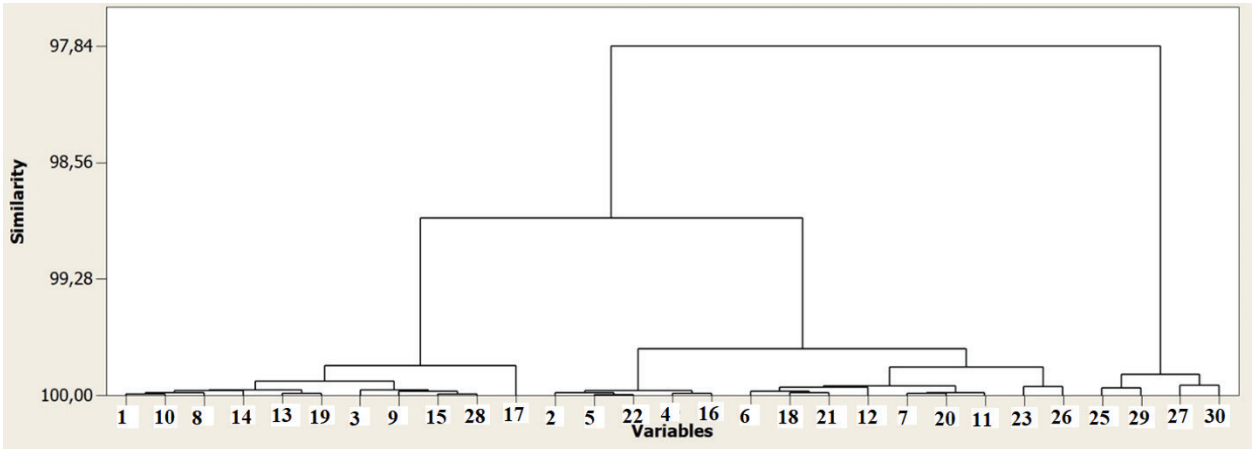

1C

Figure 1: Dendogram results of HRM analysis. (1A: Before transplantation, 1B: After transplantation, 1C: Donors)

Table 4: Nucleic acid pools of donors

D-POOL 1	D-POOL 2	D-POOL 3	D-POOL 4
Firmicutes	Firmicutes	Firmicutes	Firmicutes
Bacteroidetes	Bacteroidetes	Bacteroidetes	Bacteroidetes
Proteobacteria	Actinobacteria	Proteobacteria	Proteobacteria
Actinobacteria	Proteobacteria	Actinobacteria	Actinobacteria
Tenericutes	Verrucomicrobia	Verrucomicrobia	Tenericutes
Cyanobacteria	Tenericutes	Tenericutes	Verrucomicrobia
Verrucomicrobia	Cyanobacteria	Elusimicrobia	Kiritimatiellaeota
	Kiritimatiellaeota	Cyanobacteria	Synergistetes

D: Donor

Table 5: Before transplant nucleic acid pools of recipients

BT-POOL 1	BT-POOL 2	BT-POOL 3	BT-POOL 4	BT-POOL 5	BT-POOL 6
Firmicutes	Firmicutes	Firmicutes	Firmicutes	Firmicutes	Firmicutes
Bacteroidetes	Bacteroidetes	Bacteroidetes	Bacteroidetes	Proteobacteria	Proteobacteria
Proteobacteria	Proteobacteria	Verrucomicrobia	Proteobacteria	Bacteroidetes	Bacteroidetes
Verrucomicrobia	Verrucomicrobia	Proteobacteria	Actinobacteria	Actinobacteria	Actinobacteria
Actinobacteria	Actinobacteria	Actinobacteria	Tenericutes	Epsilonbacteraeota	
Tenericutes	Patescibacteria	Cyanobacteria			
Patescibacteria		Synergistetes			
Epsilonbacteraeota		Patescibacteria			
		Fusobacteria			

BT: Before transplant

Table 6: After transplant nucleic acid pools of recipients

AT-POOL 1	AT-POOL 2	AT-POOL 3	AT-POOL 3	AT-POOL 4	AT-POOL 5
Proteobacteria	Firmicutes	Bacteroidetes	Firmicutes	Firmicutes	Firmicutes
Bacteroidetes	Proteobacteria	Firmicutes	Bacteroidetes	Proteobacteria	Bacteroidetes
Firmicutes	Bacteroidetes	Proteobacteria	Synergistetes	Actinobacteria	Proteobacteria
Actinobacteria	Actinobacteria	Verrucomicrobia	Proteobacteria	Bacteroidetes	Actinobacteria
	Epsilonbacteraeota	Actinobacteria	Actinobacteria		Verrucomicrobia
			Fusobacteria		

AT: After transplant

The microbiota of the donors was found to be similar to the microbiota flora of the patients before HSCT. Intestinal microbiota of donors consisted of *Firmicutes* phylum with a ratio of 70% and *Bacteriodetes* phylum being 24%. Patients' pretransplant intestinal microbiota consisted of *Firmicutes* phylum, *Bacteriodetes,* and *Proteobacteria* phylum, with a ratio 63%, 24%, and 11%, respectively. Posttransplant intestinal microbiota consisted of 54% *Firmicutes* phylum, 25% *Bacteriodetes*, and 20% *Proteobacteria* phylum. The change in intestinal microbiota was found statistically significant when pre-transplant and post-transplant samples were compared were compared (p:0.02). The posttransplant samples showed a decrease in the *Roseburia* (p:0.01), *Bifidobacterium* (p:0.05), *Faecalibacterium* (p:0.04), and *Dorea* (p:0.05) genus (Figure 2). An increase was observed in *Lachnoclostridium* (p:0.1), *Bacteroides* (p:0.1), and *Veillonella* (p:0.3) genus (Figure 3).

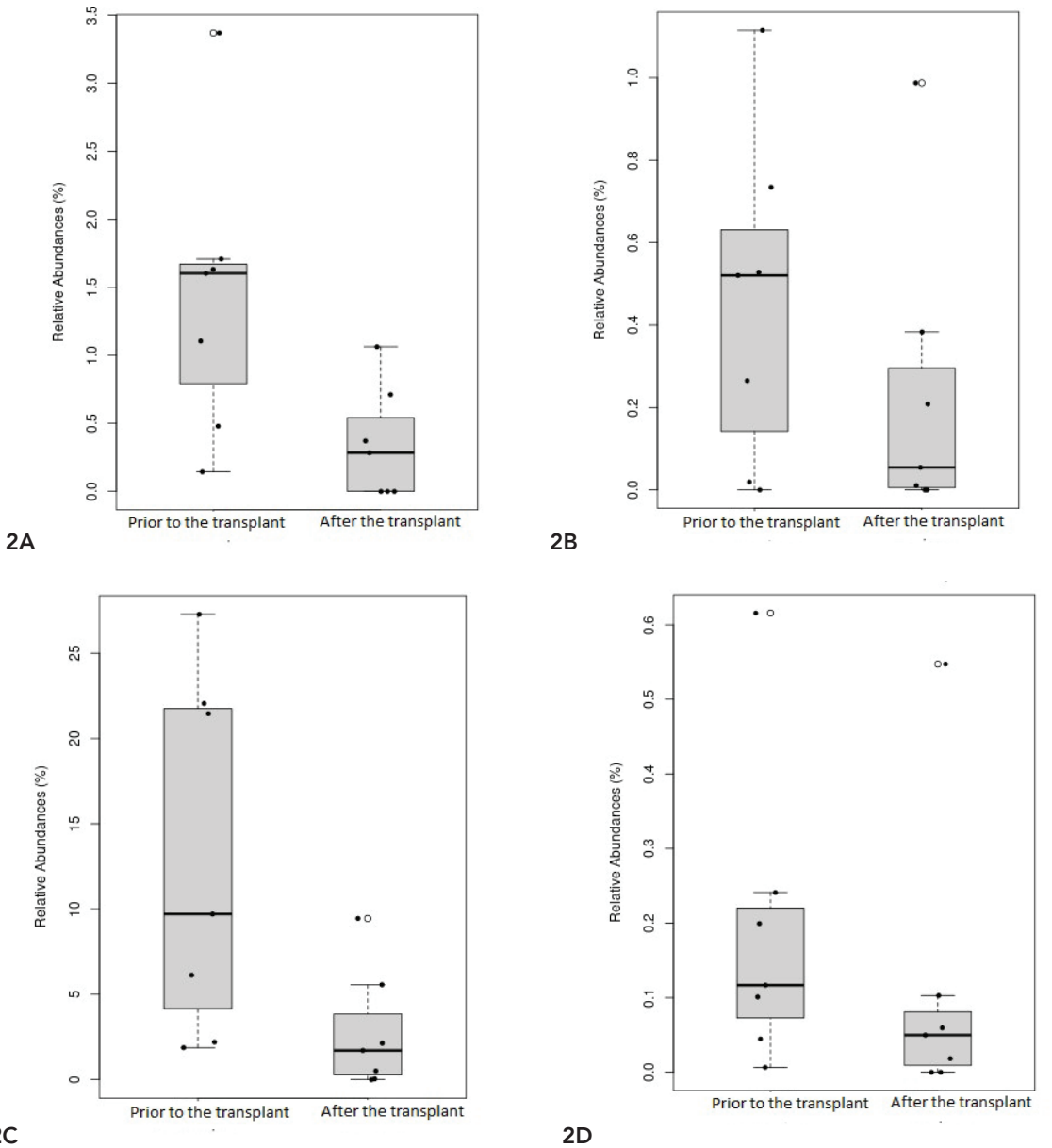

Figure 2: Decreased genus in the microbiota during engraftment. (2A: Genus of *Roseburia* levels prior to and after the transplant, 2B: Genus *of Bifidobacterium* levels prior to and after the transplant, 2C: Genus of *Faecalibacterium* levels prior to and after the transplant, 2D: Genus of *Dorea* levels prior to and after the transplant)

In patients who developed aGVHD, an increase in *Enterococcus* genus (p:0.3) was observed (Figure 4), which was not statistically significant. Neither was any significant difference detected in *Streptococcus* (p:0.3) genus (Figure 5).

DISCUSSION

The intestinal microbiota build up key factors for the regulation of intestinal homeostasis, the immune response and the pathogenesis of aGVHD after allo-HSCT. It has been shown that the intestinal microbiota changes and diversity of intestinal microbiota decreases during allo-HSCT. This condition is known as dysbiosis (11-13). Taur et al. showed an association between engraftment and low intestinal diversity (8). The first hypothesis that suggested that intestinal microbiota affected the GVHD dates back to the early 1970s (14, 15). An animal study showed the *Lactobacillus* species decrease after HSCT in mice, and this was associated with GVHD (16). Mathewson et al. reported a butyrate level decrease in mice intestinal epithelial cells after allo-HSCT. This condition

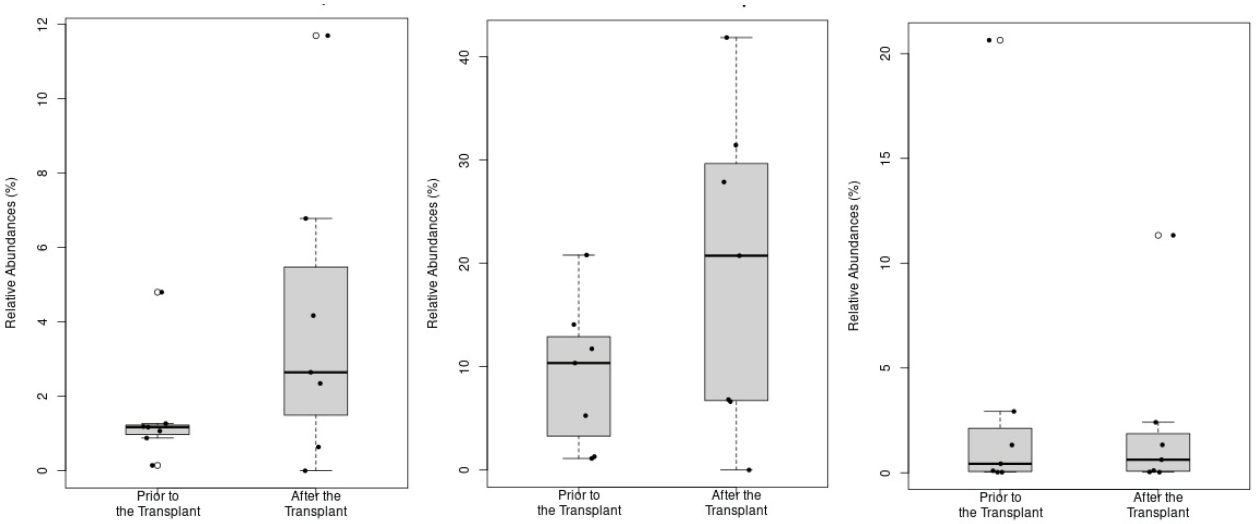

Figure 3: Increased genus in the microbiota during engraftment. (3A: Genus of *Lachnoclostridium* levels prior to and after the transplant, 3B: Genus of *Bacteroides* levels prior to and after the transplant, 3C: Genus of *Veillonella* levels prior to and after the transplant)

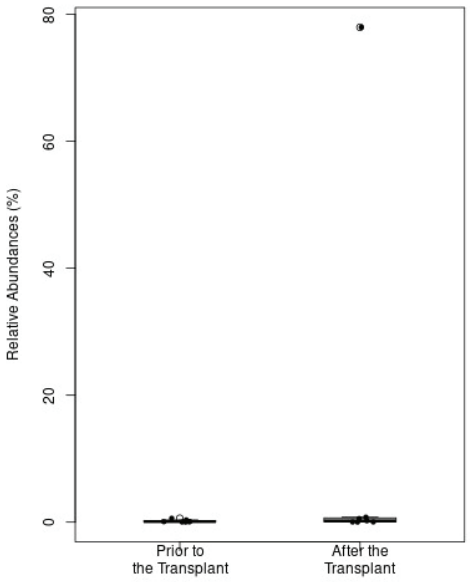

Figure 4: Levels of *Enterococcus* before and after transplantation

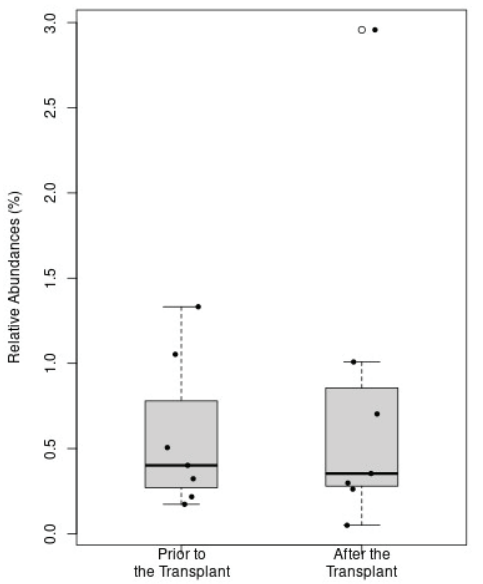

Figure 5: Levels of *Streptococcus* before and after transplantation

might increase the intestinal damage and may be related with the development of GVHD (17).

We also showed a statistically significant decrease in the reading values before and after HSCT. In addition, microbiota analysis of donors was shown to be similar to pre-transplant microbiota analysis of patients. After allo-HSCT, a decrease was shown in the *Lactobacillus* species. In the patient who developed gastrointestinal aGVHD, an increase in *Enterococcus* and *Clostridium* species was detected. This patient succumbed to aGVHD. Indeed, GIS aGVHD progresses more severely compared to other organ involvements and inflammation in GIS plays important role in the onset and exacerbation (18).

Mathewson et al reported a decrease of particularly SCYA-producing anaerobes after allo-HSCT (17). In the course of HSCT, loss of *Clostridia* species was detected, known as SCFA producer from the nutrient fibers (19). In some mouse studies a decrease in the diversity of the health-improving *Faecalibacterium* and a high diversity of *Enterococci* have been reported, especially at the onset of GVHD (20). In this study, a decrease in the *Clostridia* species has been observed in stool samples after transplantation, however, no statistical significance was detected. An increase was detected in *Enterococcus* in patients who developed GVHD after transplantation, and this increase was not found statistically significant.

In one study, the relative increase of the *Lachnospiraceae* species and the decrease in *Blautia* species were found to be associated with a decrease in mortality rates from GVHD (21). Another study highlighted that *Enterococcus* species had critical importance in the pathogenesis of GVHD (22). In our study, we also detected a decrease in the L*achnospiraceae* and *Blautia* species in the post-transplant samples, however no association was detected between GVHD and mortality.

Citrobacter murliniae, Klebsiella pneumoniae, and *Enterobacter cloacae*, which are known as the hospital pathogens, are highly important for the risk of infection in HSCT. *Enterococcus spp, Citrobacter spp,* andother members of *Enterobacteriaceae* family, such as *Enterobacter spp and Klebsiella spp*, are the most opportunistic members of the human intestinal microbiota. *Citrobacter, Enterococcus, Klebsiella,* and *Enterobacter* species are well-known possible sources of nosocomial infections and have been reported as the cause of morbidity and mortality (23).

The relative increase of the saccharolytic commensals such as Blautia, or *Fusobacterium nucleatum* are risk factors for localized mucosal damage in allogeneic HSCT patients. Pretransplant and posttransplant so called "pathobiome" cannot support immunological recovery in HSCT patients. Pathobiome may become an 'adapted pathogenic community' (24).

It is thought that the commensal flora may expand or GIS metabolome may be controlled by probiotics. Non-pathogenic microorganisms may change microbiota. Enrichment of organisms may prevent dysbiosis, bacteremia, and sepsis after allo-HSCT. A better understanding of the human ecosystem may allow the recognition of microbiota composition. This information may be used as a biomarker in the future, and may allow earlier intervention during HSCT, especially in conditions with risk prediction for steroid-resistant GVHD.

In general, microbiota-based therapeutics show great promise for the prevention and treatment of GVHD in HSCT patients. It is important to conduct further research on the development of targeted and individualized dysbiosis prevention and treatment regimens applicable to HSCT patients.

In our study, we showed that the microbiota changed after allo-HSCT. We think that the relationship between GVHD and clinical course can reach statistical significance by increasing the number of patients. The present study is a preliminary study that forms the basis of this field, and more reliable information will be obtained by conducting a greater number of studies.

Ethics Committee Approval: This study was approved by the local ethics committee of Istanbul University, Istanbul Faculty of Medicine (Date: 19.07.2019, No: 146386).

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- F.S.O., S.B.K., E.E.G., M.O.Ö., Z.G.; Data Acquisition- E.E.G.; Data Analysis/Interpretation- F.S.O., S.B.K., U.S., Z.A.; Drafting Manuscript- E.E.G., F.S.O., S.B.K., Z.A.; Critical Revision of Manuscript- F.S.O., E.E.G., S.B.K., M.O.Ö.; Approval and Accountability- E.E.G., F.S.O., M.O.Ö.; Supervision- F.S.O., Z.A., S.B.K., M.O.Ö.

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