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Original Scientific Paper

MATERNAL GUT MICROBIOTA IN PREGNANCIES RESULTING IN DOWN SYNDROME NEWBORNS – A PILOT STUDY

Mehmet Hursitoglu[1,2], Alev Kural[3], Sibel Kuras[4], Esra Akdeniz[5], Salim Sezer[6], Sema Suzen Caypinar[6], Cemal Kazezoglu[7], Bulent Yaprak[1], Faruk Karandere[1] and Hamit Zafer Guven[8]

[1]Internal Medicine Department, Bakirkoy Dr. Sadi Konuk Training & Research Hospital, University of Health Sciences, Istanbul, Turkey;
[2]Internal Medicine Department, Basaksehir Cam & Sakura Hospital, University of Health Sciences, Istanbul, Turkey;
[3]Biochemistry Department, Bakirkoy Dr. Sadi Konuk Training & Research Hospital, University of Health Sciences, Istanbul, Turkey;
[4]Biochemistry Department, Hamidiye Medical Faculty, University of Health Sciences, Istanbul, Turkey;
[5]Department of Medical Education, Marmara Medical Faculty, Marmara University, Istanbul, Turkey;
[6]Department of Gynecology and Obstetrics, Kanuni Sultan Suleyman Training & Research Hospital, University of Health Sciences, Istanbul, Turkey;
[7]Biochemistry Department, Kanuni Sultan Suleyman Training & Research Hospital, University of Health Sciences, Istanbul, Turkey;
[8]Gynecology and Obstetrics Department, Bakirkoy Dr. Sadi Konuk Training & Research Hospital, University of Health Sciences, Istanbul, Turkey

SUMMARY – Down syndrome (DS) is one of the main genetic abnormalities of newborns. Therefore, prenatal diagnosis of this syndrome is of paramount importance to the family and the community. The microbiota system is important in early brain development. We tried to study and compare gut microbiota (GM) composition in pregnancies that resulted in DS neonates with pregnancies that resulted in healthy children. The study population consisted of 21 pregnant women having delivered DS newborns (group 1) and 22 pregnant women who had given birth to healthy newborns (group 2). The GM composition was determined and compared between the two groups. There were no significant age and gestational age differences between the two groups ($p>0.005$ both). Regarding GM analysis, microorganisms of the families *Clostridiaceae* and *Pasteurellaceae* were more abundant in the group of women having delivered DS neonates than the group of women having delivered healthy newborns ($p<0.05$). The results of our pilot study showed that the GM system might have a role in the pathophysiology of DS. The GM changes may be used in the prenatal diagnosis and prevention of this syndrome. Further studies are needed in this field.

Key words: *Brain; Down syndrome; Microbiota; Pregnancy; Outcome; Screening*

Introduction

Most neonatal deaths are due to genetic disorders. More than one-fourth of pediatric hospital admissions are due to these genetic disorders. So, detection and management of these risky pregnancies are important from the viewpoint of both the mother and the baby.

Correspondence to: *Assoc. Prof. Mehmet Hursitoglu, MD*, Internal Medicine Department, Bakirkoy Dr. Sadi Konuk Training & Research Hospital, University of Health Sciences, 34100 Istanbul, Turkey
E-mail: hursitoglum@yahoo.com; mehmet.hursitoglu@sbu.edu.tr
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Measuring maternal alpha-fetoprotein, human chorionic gonadotropin, unconjugated estriol and inhibin-A levels at 14-18 weeks of gestation (known as a quadruple screening test) may help in determining adverse pregnancy outcomes (Down syndrome [DS], neural tube defects, etc.) and advising further investigations (such as amniocentesis) and follow-up[1,2]. The human microbiome, i.e. trillions of bacteria that reside on or inside the body, has been implicated in the pathophysiology of many diseases[3,4]. Gut microbiota (GM; complex bacterial community located in the gastrointestinal tract) influences the development and diseases of the central nervous system[3]. Recent studies show that maternal microbiota affects pregnancy outcome and/or infant health[5-8]. During the first trimester, GM composition is similar to that in non-pregnant women. Later, the levels of *Faecalibacterium* and microorganism diversity decrease, whereas the levels of *Proteobacteria* and *Actinobacteria* increase[9]. In a study by Crusell *et al.*[10], the GM composition of pregnant women with gestational diabetes mellitus was similar to that in non-pregnant women with type 2 DM both during pregnancy and after delivery. To our knowledge, there is no study of GM composition in women with DS pregnancies. There is also only one study of GM composition in DS persons, conducted by Biagi *et al.*[11]. In this study, DS persons had increased *Parasporobacterium* and *Sutterella* species in their GM analysis. Additionally, the abundance of *Sutterella* microorganisms was significantly correlated with the Aberrant Behavior Checklist total score[11].

Therefore, we tried to study and compare the GM composition between pregnancies having ended in giving birth to DS newborns and pregnancies with healthy newborns.

Materials and Methods

This prospective study was approved by the institutional Ethics Board (SBU Istanbul Bakırkoy Training and Research Hospital, approval no. 2018-04-16) and conducted according to the Declaration of Helsinki. It was performed in the Prenatal Diagnosis Center, Obstetrics and Gynecology Department, Istanbul Kanuni Sultan Suleyman Training & Research Hospital, University of Health Sciences, Turkey. Pregnant women having undergone karyotyping of fetal DS (by chorionic villous, amniocentesis, or cordocentesis sampling, according to gestational age) due to advanced maternal age or elevated risk of fetal aneuploidies based on prenatal screening were invited to participate in this prospective study. Those who agreed and gave their written consent were enrolled in the study. According to karyotyping results, 21 pregnant women were included in group 1 (trisomy [T]21 fetus pregnancy). The other group (control group) consisted of 23 age-matched pregnant women with chromosomally normal fetuses.

Inclusion criteria for both groups were age ≥18 years, singleton pregnancy, and availability of karyotyping results. Exclusion criteria were inability to give written consent (both groups), presence of other fetal chromosomal anomalies (group 2), using medications that might affect GM composition (both groups), presence of acute and/or chronic infection or inflammation (both groups), and presence of any malignancies (both groups).

Upon inclusion in the study, stool samples (for GM analysis) were obtained at the hospital and kept at -80 °C until analysis. Each sample was given a special code (to conceal the participant's identity). Two participants from group 1 (withdrawal of their acceptance) and one participant from group 2 (due to using herbs available in markets) were excluded from the study. So, the final analysis included 43 participants (21 in group 1 and 22 in group 2).

Microbiota analysis

Stool sample collection and DNA isolation

From each participant, about 1 g or 1 mL stool was self-collected into a 15-mL container (prefilled with 9 mL DNA/RNA Shield). As mentioned above, the collected samples were kept in our laboratory at -80 °C until analysis. Following the manufacturer's instruction, isolation and genomic purification of stool microbiota samples were done with the ZymoBIOMICS™ DNA Microprep Kit (Zymo Research, Irvine, CA, USA). After this stage, the isolated DNA samples were sent to the Zymo Research Central Laboratory (CA, USA) for further analysis. This analysis was done by the service procurement method. This laboratory was completely blinded to the sample groups.

Targeted library preparation

The DNA samples were prepared for targeted sequencing with the Quick-16S™ NGS Library Prep

Kit (Zymo Research, Irvine, CA, USA). These primers were custom-designed by Zymo Research to provide the best coverage of the 16S gene while maintaining high sensitivity. The primer sets used in this project were Quick-16S™ Primer Set V3-V4 (Zymo Research, Irvine, CA, USA).

The sequencing library was prepared using an innovative library preparation process in which polymerase chain reactions (PCR) were performed in real-time PCR machines to control cycles and therefore limit PCR chimera formation. The final PCR products were quantified with qPCR fluorescence readings and pooled together based on equal molarity. The final pooled library was cleaned up with the Select-a-Size DNA Clean & Concentrator™ (Zymo Research, Irvine, CA, USA), then quantified with TapeStation® (Agilent Technologies, Santa Clara, CA, USA) and Qubit® (Thermo Fisher Scientific, Waltham, WA, USA).

Control samples

The ZymoBIOMICS® Microbial Community Standard (Zymo Research, Irvine, CA, USA) was used as a positive control for each DNA extraction if performed. The ZymoBIOMICS® Microbial Community DNA Standard (Zymo Research, Irvine, CA, USA) was used as a positive control for each targeted library preparation. Negative controls (i.e. blank extraction control, blank library preparation control) were included to assess the level of bioburden carried by the wet-lab process. Sequencing: the final library was sequenced on Illumina® MiSeq™ with a v3 reagent kit (600 cycles). The sequencing was performed with >10% PhiX spike-in.

Bioinformatic analysis

Unique amplicon sequences were inferred from raw reads using the DADA2 pipeline[12]. Chimeric sequences were also removed with the DADA2 pipeline. Taxonomy assignment was performed using Uclust from QIIME v.1.9.1. Taxonomy was assigned with the Zymo Research Database, a 16S database that is internally designed and curated, as reference.

Composition visualization, alpha diversity, and beta diversity analyses were performed with QIIME v.1.9.1[13]. If applicable, a taxonomy with significant abundance among different groups was identified by LEfSe using default settings[14]. Other analyses such as heatmaps, Taxa2SV_deomposer, and PCoA plots were performed with internal scripts.

Statistical analyses

Alpha diversity of GM was measured by the number of Operational Taxonomic Units (OTUs), the Shannon diversity index, the Chao1 index, Faith's phylogenetic diversity (PD) whole tree. An input phylogenetic tree was used for calculating beta diversity. Hypothesis testing in microbial taxa was performed by comparing alpha and beta diversity indices. Depending on the normal distribution or non-distribution of data, the t-test or related non-parametric test (Mann-Whitney U test) was used.

The relative abundance of single taxa within the GM of every subject was expressed as a percentage of the whole number of bacteria detected by metagenomic analyses. Significant differences in the phylum- or genus-level abundance between DS pregnant women and healthy pregnant controls were assessed by Mann-Whitney U tests and corrected for multiple comparisons using the Benjamini-Hochberg method when appropriate.

Our hypothesis was to test the association between microbiome and host, i.e. whether the microbiome composition or 'dysbiotic' microbiome was linked to the health or disease of the host. For example, in our research, we hypothesized that dysbiosis was associated with the presence or absence of DS resulting pregnancies.

The Spearman correlation coefficient evaluated relationships between serologic variables and microbial biodiversity variables (Shannon index). We used Spearman correlation, which is robust to nonlinear relationships and outliers.

The OTU data were used to calculate the index of biodiversity Chao1 and to perform beta diversity analysis with the Principal Coordinate Analysis (PCoA) method based on unweighted UniFrax.

The overall fecal microbiota composition, in terms of interindividual variability, was compared between DS and normal pregnancies using PERMANOVA.

In the sample collection period, power analysis is conducted to estimate how many samples are needed to provide sufficient power (e.g., 80%) to correctly conclude on a difference between the groups. We estimated a sample size of 21 for each group for an effect size of 0.9 to compare beta diversity between the

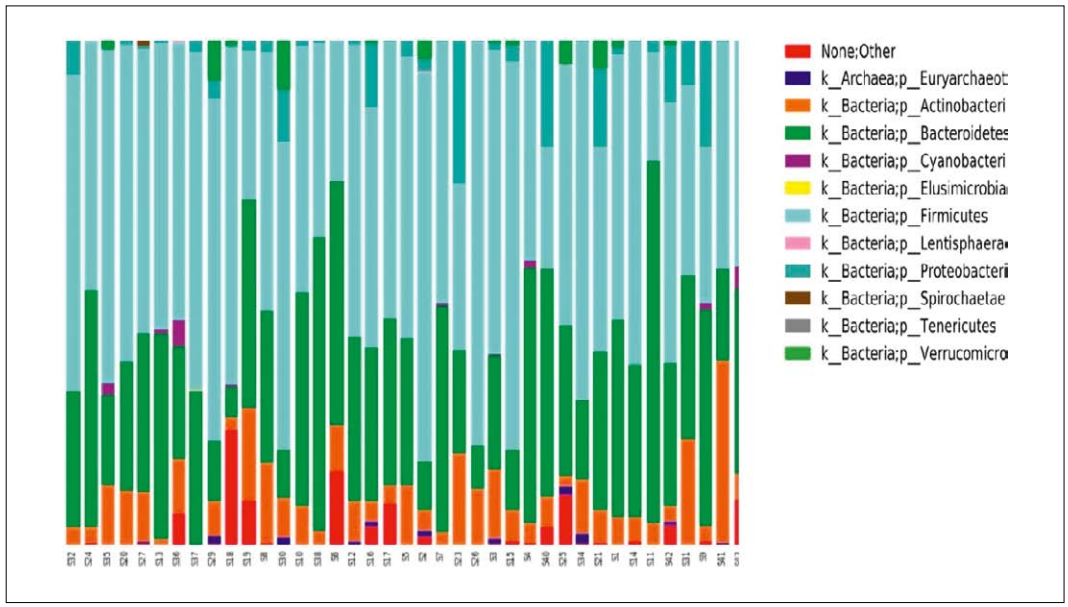

Fig. 1. A stacked bar chart showing phylum level abundance profiles of the enrolled women.

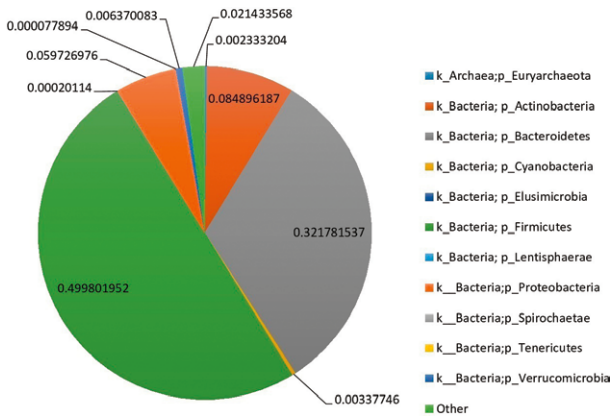

Fig. 2. Average phylum level abundance profiles of gut microbiota of the enrolled Down syndrome pregnancies.

groups. We used GPower 3.1 to conduct power analysis. As in the study by Bingula *et al.*[15], a sample size of 20 participants in each group was sufficient for microbiota composition comparison. All other analyses were done using R version 3.6.2, considering p≤0.05 as significant. Vegan, micropower R, and UniFrac R packages were used on analyses[16].

Results

To highlight the possible GM signatures of DS resulting pregnancies, the microbiota structure from 21 pregnant women having delivered DS newborns (group 1), mean age 34.08±5.48 (min-max: 20-46) years were enrolled in the study and compared with that from 22 pregnant women having given birth to normal children (group 2), mean age 32.40±7.64 (min-max: 20-42) years. Gestational age was 21.40±5.02 (min-max: 12-31) weeks in group 1 and 21.70±5.30 (min-max: 16-32) weeks in group 2. According to independent samples t-test results, there were no significant differences in the mean age and mean gestational age between the two groups (p=0.21 and p=0.96, respectively). Thus, any age- or gestational age-related effect on microbiota structure was excluded.

Microbiota analysis

Taxonomy composition graphics show microbial composition at different taxonomic levels, i.e. at phylum and family levels (Fig. 1). Numbers were given to each OTU included in the study. Numbers between S32 and S2 indicate women giving birth to children with DS, while numbers between S7 and S39 indicate women giving birth to normal children.

The relative abundance of phylum-level assigned OTUs is reported in pregnancies that resulted in DS newborns and in normal pregnancies (Figs. 2 and 3, respectively). Colors were assigned for all phyla detected. According to our data, the GM of DS persons was largely predominated by *Firmicutes* (relative abun-

dance (rel. ab.) 49.9%), *Bacteroidetes* (rel. ab. 32.2%), and *Actinobacteria* (rel. ab. 8.4%). With relative abundance values below 5%, *Elusimicrobia, Spirochaetes, Tenericutes, Lentisphaerae, Euryarchaeota, Cyanobacteria*, and *Verrucomicrobia* were largely subdominant phyla. The GM of normal pregnant women was largely predominated by *Firmicutes* (rel. ab. 55.4%), *Bacteroidetes* (rel. ab. 29.8%), and *Actinobacteria* (rel. ab. 7.4%). With relative abundance values below 5%, *Elusimicrobia, Spirochaetes, Tenericutes, Lentisphaerae, Euryarchaeota, Cyanobacteria, Proteobacteria*, and *Verrucomicrobia* were largely subdominant phyla.

The most represented families in DS gut microbial communities were *Prevotellaceae* (rel. ab. 25.7%), *Lachnospiraceae* (rel. ab. 24.1%), *Ruminococcaceae* (rel. ab. 16.8%), and *Bifidobacteriaceae* (rel. ab. 5.2%). The most represented families in normal gut microbial communities were *Lachnospiraceae* (rel. ab. 25.6%), *Prevotellaceae* (rel. ab. 24.3%), *Ruminococcaceae* (rel. ab. 19.4%), and *Bifidobacteriaceae* (rel. ab. 4.5%).

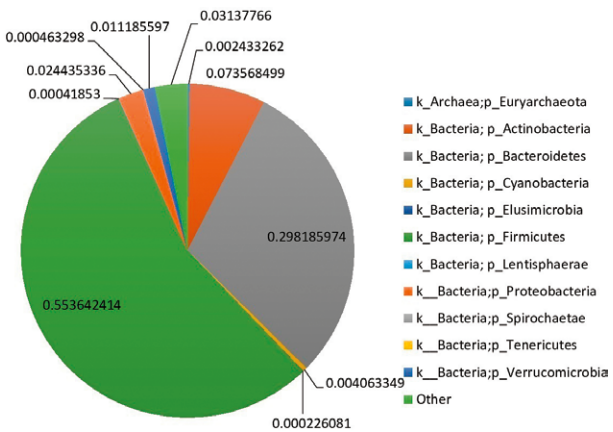

Fig. 3. Average phylum level abundance profiles of gut microbiota of the enrolled normal pregnancies.

The lower taxonomic levels are not shown due to limited space; however, the two groups were compared to the lowest taxon as possible based on the Linear discriminant analysis Effect Size (LEfSe), which is a tool developed by the Huttenhower group to find biomarker bacteria between two or more groups based on relative abundances.

The LefSE chart creates a chart with bars that represent the effect size (linear discriminant analysis, LDA) for a particular taxon in a particular group (Fig. 4). The length of the bar represents a log10 transformed LDA score. The colors indicate which taxon is more common than in the other group. Here, the taxa that showed significance in normal and DS resulting pregnancy groups are shown with red and purple bars, respectively. According to the LefSe chart, *Succinivibrionaceae* (family) and *Lachnoclostridium_Roseburia* (genus) were more dominant in normal than in DS pregnancies, whereas *Pasteurellales* (order), *Clostridiaceae* (family), *Pasteurellaceae* (family), *Clostridium* (genus), *Intestinibacter* (genus), *Terrisporobacter* (genus) and *Haemophilus* (genus) abundances were significantly higher in the women with DS resulting pregnancies than in the women with normal pregnancies.

Alpha diversity and beta diversity comparisons

Change in microbial diversity of the intestinal microbiome is an important indicator for most diseases.

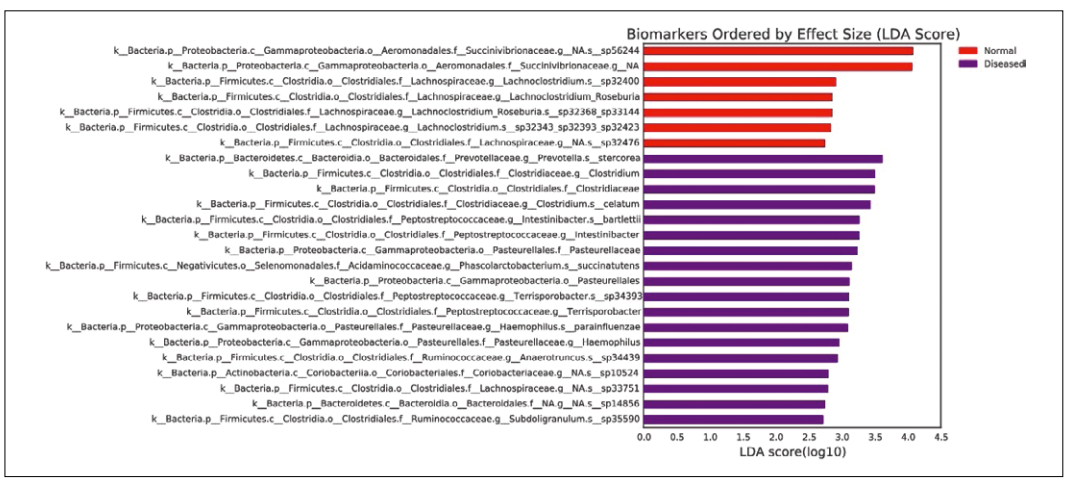

Fig. 4. Linear discriminant analysis Effect Size (LEfSe) graph.

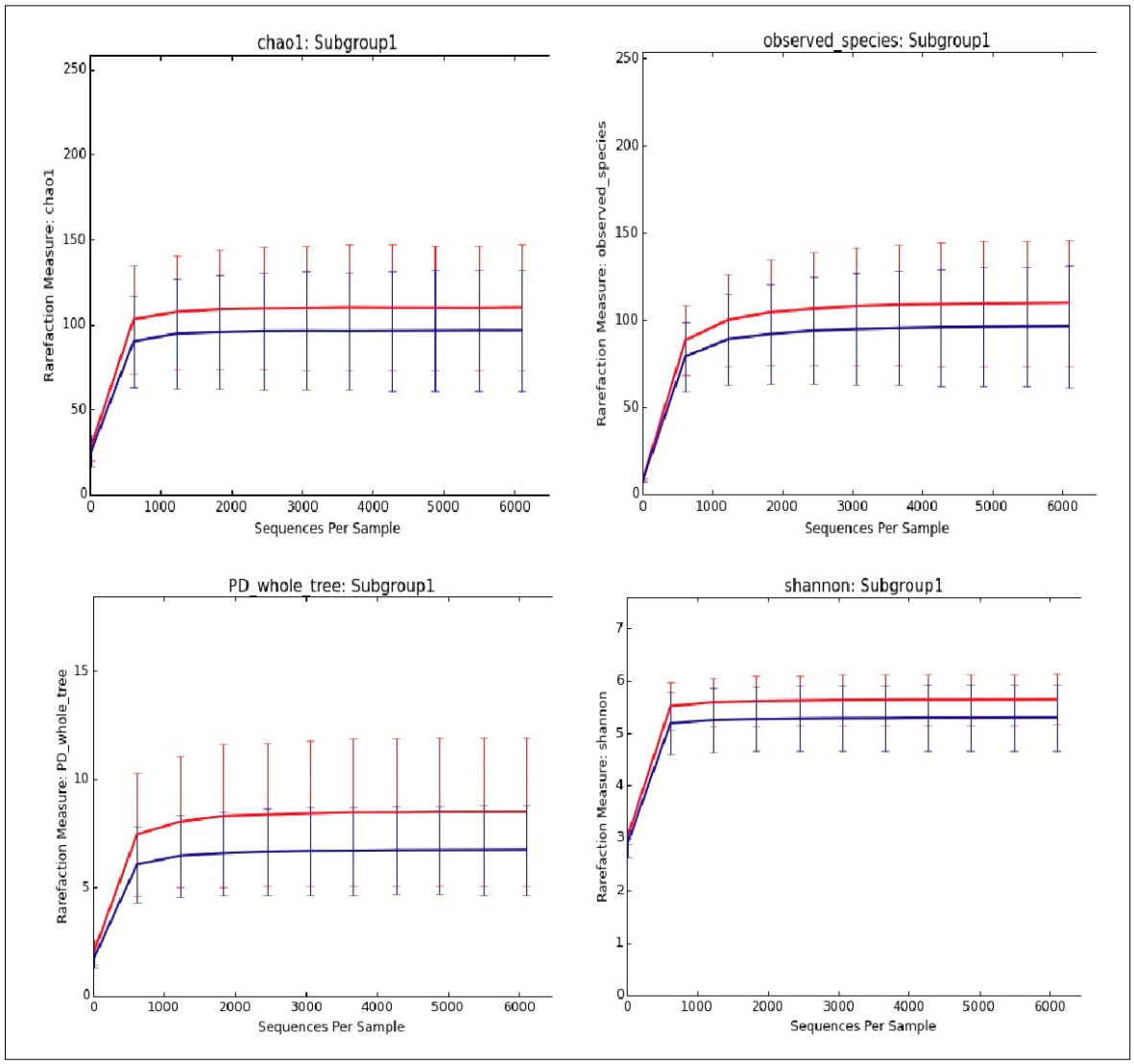

Fig. 5. Average gut microbiota biodiversity of the 43 stool samples analyzed by 16S rRNA microbial profiling metagenomics techniques of the patients with normal pregnancy (blue) and Down syndrome resulting pregnancy (red). The curves represent the average Chao1 index (up left), observed species (up right), PD whole tree (down left) and Shannon index (down right), corresponding to the number of Operational Taxonomic Units (OTUs), at increasing sequencing depth.

Therefore, we compared these conditions with microbial diversity in women with DS and normal resulting pregnancy. Superimposition of the rarefaction curves of different α-diversity metrics (PD whole tree, observed OTUs, the Chao1 measure of microbial richness, and the Shannon index of biodiversity) are shown in Figure 5. The sequencing depths used in the analysis were 10, 619, 1228, 1837, 2446, 3055, 3664, 4273, 4882, 5491, 6100 curves that reached the plateau, approximating the saturation level, after 1228 reads.

Differences between the Shannon, Chao1, observed species, and PD whole tree indices were examined for each sequencing. According to the results of the Mann-Whitney U test, pregnant women with DS had more diversity (measured by Shannon index) and richness (measured by Chao1 index) than normal ones ($p<0.001$). In other words, the pregnant women with DS had more OTU members and more skewed distributions.

Beta diversity is a measure of microbial diversity differences between samples. The PCoA graph created

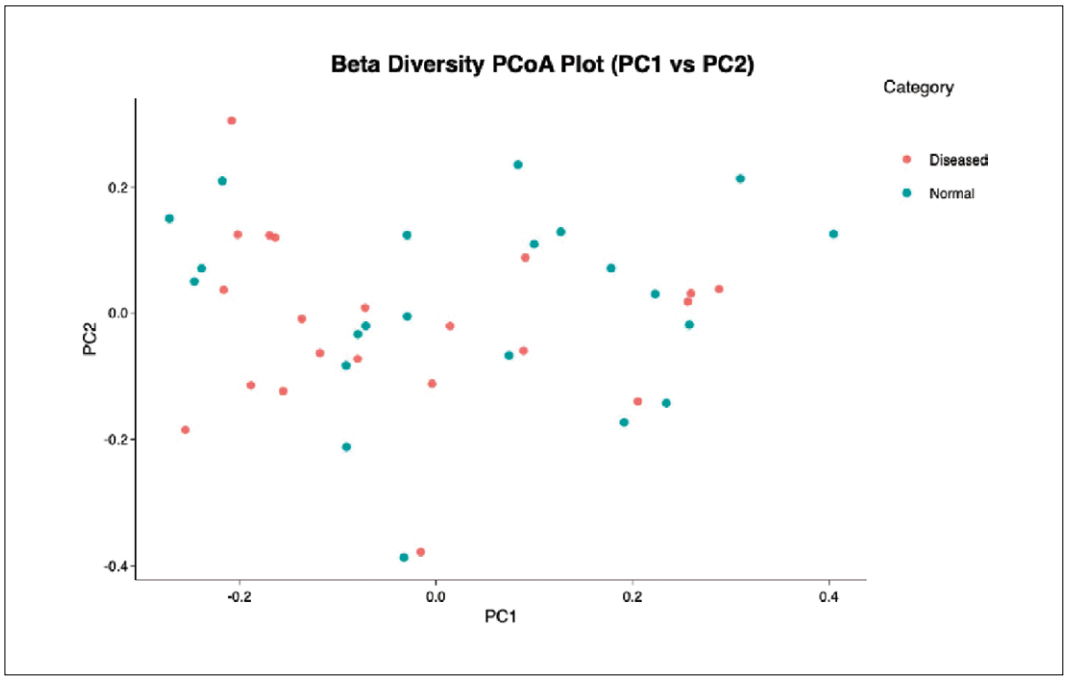

Fig. 6. Gut microbiota in cases and controls: comparison of the overall fecal microbiota composition, represented with 2D Principal Coordinate Analysis (PCoA) scatterplot based on the Bray-Curtis distance matrix.

Table 1. Clinical metadata: main characteristics and comparison of diseased and control groups

	Normal pregnancy (n=22)					Down syndrome resulting pregnancy (n=21)					
	M	SD	M	IQR	Min; max	M	SD	M	IQR	Min; max	p
Age (yrs)	32.4	7.64	32.5	12.5	20; 46	34.8	5.48	37	6	20; 42	0.21*
Gestational age	21.7	5.30	19.5	8	16; 32	21.4	5.02	22	7	12; 31	0.96*

M = median; SD = standard deviation; IQR = interquartile range; 95% Bias Corrected and Accelerated (BCA) bootstrap confidence interval for median differences, *independent samples t-test

using the matrix of the matched distance between the Bray-Curtis difference and the samples calculated is illustrated in Figure 6. Each point in the figure represents the entire microbial composition profile. Thus, the samples with similar microbial composition profiles are close to each other.

There was no significant difference in fecal microbiota composition between the women with DS pregnancies and normal pregnancies in terms of beta diversity (PERMANOVA, p=0.104). In addition, the PCoA of β-diversity comparison using Bray Curtis distances did not reveal a significant separation of microbial communities between the DS and normal resulting pregnancies (Fig. 6).

Discussion

Down syndrome is one of the most common incurable chromosome abnormalities in humans[17]. Amniocentesis (an invasive test) is required for a definitive diagnosis of DS during pregnancy. To the best of our knowledge, this issue has not been evaluated and studied in DS resulting pregnancies. GM has a role in the gut-brain axis. Evidence is emerging about nutrition and GM in the development and function of the nervous system[18,19]. Altering GM composition (by diet, probiotics, etc.) may prevent the symptoms (or their severity) of development of some neurodegenerative diseases[20]. So, studying this issue in DS resulting pregnancies is of paramount importance. Even in DS per-

sons, there is only one study evaluating their GM composition. In this sole study, *Parasporobacterium* and *Sutterella* microorganisms were more abundant in DS resulting pregnancy group (in comparison to the healthy pregnancy group)[11]. So, whether this different GM composition of DS persons is the cause or the result of this disease needs to be studied in-depth[19]. Our study tried to compare the GM composition between women with DS resulting pregnancies and women who had delivered healthy babies. One of the essential measures of ecologic diversity is the alpha diversity curve. Pregnant women with DS had more diversity (measured by Shannon index) and richness (measured by Chao1 index) than normal ones ($p<0.001$). In other words, the women with DS pregnancy had more OTU members and more skewed findings. On the other hand, beta diversity did not reveal a significant separation of microbial communities between the DS and normal resulting pregnancies (PERMANOVA, $p>0.05$).

Analyzing the Cladogram chart, which helps see the tax difference between the groups, showed the families *Clostridiaceae, Pasteurellaceae* and *Pasteurellales* to be more abundant in DS resulting pregnancy group than in the group with normal babies. Studies are ongoing to use GM dysbiosis (microbiomarkers) in the diagnosis and/or treatment of inflammatory bowel disease[21]. This is the case in Parkinson's disease as well[22]. Whether these significant findings of GM dysbiosis could be used in the diagnosis and/or prevention of DS during pregnancy needs to be further studied.

Limitations

One of the important limitations of this pilot study was its cross-sectional design. Pre-conception or early conception phase measures of the study parameters might yield more useful information about the role of these parameters in pregnancy outcome, and *vice versa*. Still, the data obtained in our pilot study will be a pathfinder for such detailed studies in this important and challenging field.

Conclusions

The results of this pilot study are promising. There was a diversity in the GM of women with DS resulting pregnancies. This diversity of the GM system may have a role in the pathophysiology of DS. Whether we could use this system in the diagnosis and/or prevention of DS during the prenatal period needs to be studied in-depth.

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Sažetak

MAJČINA CRIJEVNA MIKROBIOTA U TRUDNOĆAMA IZ KOJIH SU ROĐENA DJECA S DOWNOVIM SINDROMOM – PROBNO ISPITIVANJE

M. Hursitoglu, A. Kural, S. Kuras, E. Akdeniz, S. Sezer, S.S. Caypinar, C. Kazezoglu, B. Yaprak, F. Karandere i *H.Z. Guven*

Downov sindrom (DS) je jedna od glavnih genetskih nenormalnosti kod novorođenčadi, stoga je prenatalna dijagnostika ovoga sindroma od velike važnosti za obitelj, kao i za društvo. Sustav mikrobiote ima važnu ulogu u ranom razvoju mozga. U ovom istraživanju ispitali smo i usporedili sastav crijevne mikrobiote (CM) u trudnoćama iz kojih su rođena djeca s DS i u trudnoćama iz kojih su rođena zdrava djeca. Istraživana populacija obuhvatila je 21 trudnicu koje su rodile djecu s DS (1. skupina) i 22 trudnice koje su rodile zdravu djecu (2. skupina). Sastav CM utvrđen je i uspoređen među dvjema skupinama. Nije bilo razlike u dobi i gestacijskoj dobi između skupina ($p>0,005$ oboje). Analiza CM je pokazala da su mikroorganizmi iz porodica *Clostridiaceae* i *Pasteurellaceae* zastupljenije u skupini žena koje su rodile djecu s DS u usporedbi sa skupinom žena koje su rodile zdravu djecu ($p<0,05$). Rezultati našeg probnog ispitivanja pokazuju da bi sustav CM mogao imati ulogu u patofiziologiji DS. Promjene u CM mogle bi se rabiti u prenatalnoj dijagnostici i prevenciji ovoga sindroma. Potrebna su daljnja istraživanja u ovom području.

Ključne riječi: *Mozak; Downov sindrom; Mikrobiota; Trudnoća; Ishod; Probir*