ORIGINAL RESEARCH—BASIC

Detection of Human Y Chromosome and the *SRY* Gene in Fecal Samples of Female Patients Following Fecal Microbiota Transplantation

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BACKGROUND AND AIMS: We have postulated that fecal microbiota transplantation (FMT) is associated with transfer of microbiota from the donor and engraftment of intestinal epithelial cells in the recipient's colonic mucosa enabling the restoration of a stable microbial environment. **METHODS:** We analyzed the presence of human Y chromosome (ChrY) and sex-determining region Y (SRY) gene within total human DNA extracted from fecal samples collected from 30 donors and 22 recurrent Clostridium difficile infection (RCDI) patients before and up to 24 months after FMT. A next-generation sequencing data analysis pipeline was applied to quantify the percentage of reads aligning to human ChrY. SRY gene detection was also performed by quantitative polymerase chain reaction and droplet digital polymerase chain reaction. **RESULTS:** A significantly higher percentage of ChrY reads were identified in fecal samples of male donors as compared to female donor ($P < .0001$). Fecal samples collected from female RCDI patients who received FMT from male donors showed a significantly ($P < .05$) higher percentage of ChrY reads compared to female samples without male FMT donors. Four female patients with RCDI who received FMT from male donors showed a very large percent ChrY increase post-FMT even several months after FMT. SRY gene signal was detected by droplet digital polymerase chain reaction in 7 of the 11 fecal samples collected from the male donor pool but none from the female pool. **CONCLUSION:** These observations clearly demonstrate the presence of ChrY and SRY gene signal in stool samples collected from male patients. The presence of increased ChrY in the stool samples of female RCDI patients after FMT from a male donor suggests possible engraftment of exfoliated intestinal epithelial cells in a subset of these patients.

Keywords: Cellular engraftment; Colitis; FMT; RCDI; Y chromosome in females after FMT

Introduction

Fecal microbiota transplantation has been demonstrated to be a highly efficacious, reasonably safe, and cost-effective therapy for patients with the recurrent *C. difficile* infection.[1] Fecal microbiota transplantation (FMT) involves infusion of fecal material from a healthy human donor to the recurrent Clostridioides difficile infection (RCDI) patient's colon or the upper gastrointestinal (GI) tract with a colonoscope, endoscope, or a nasogastric tube, respectively. Its high success rate has led to consideration of its application in a variety of other GI disorders, including irritable bowel syndrome, inflammatory bowel disease, metabolic syndrome, and more recently in patients with hepatic encephalopathy.[2] The successful therapeutic outcome of FMT has been linked with altered microbial composition in fecal samples of patients with RCDI. Several studies have clearly demonstrated restoration and engraftment of bacteria from phylum *Firmicutes* that produce short chain fatty acids such as butyric acid and other metabolites. These bacteria also alter intraluminal bile acids in the intestines of FMT recipients.[3,4]

More recently, marked alterations in the fungal microbiome (mycobiome) have been reported in patients with RCDI. This fungal dysbiosis is also corrected by FMT and is characterized by donor derived fungal genera such as *Saccharomyces* and *Aspergillus* displacing *Candida* species. These observations provide evidence for potential trans-kingdom interactions between fungal and bacterial ecosystems in the microenvironment of the intestinal lumen.[5] Furthermore, in a study by Ott et al, the demonstration of FMT efficacy in 5 subjects who received sterile fecal filtrate

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Abbreviations used in this paper: **CDI, Clostridium difficile infection; ChrY, Y chromosome; dCT, delta cycle threshold; ddPCR, droplet digital polymerase chain reaction; FMT, fecal microbiota transplantation; gDNA, genomic DNA; GI, gastrointestinal; NGS, next-generation sequencing; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RCDI, recurrent Clostridium difficile infection; RNaseP, ribonuclease P; SRY, sex-determining region Y.**

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delivered by nasojejunal tube suggests an important role for bacterial phages.[6] In fact, to further complicate this very intricate and delicate microbial homeostasis in the human intestinal lumen, new emerging data suggest the presence of rich and diverse communities of bacteriophages such as C*audovirales* in both donor and post-transplant recipients associated with marked reduction in *Siphoviridae* phages in successfully treated patients with FMT.[7,8]

In spite of a large body of work that has focused on microbial (bacterial, fungal, and viral) composition of fecal samples collected from a variety of patients with GI disorders by 16 small RNA gene analysis and other metagenomic methods, several other factors such as cellular and bacterial engraftment in the FMT recipients has been poorly understood. We now know a great deal more about the clinical predictors of successful FMT such as donor characteristics, microbial profile of the recipient, effect of lyophilization of stool samples, different methods of delivery of various FMT formulations, and importance of bile acids.[9] Factors which play a role in establishing the microenvironment of the fecal sample, such as mucin and exfoliated intestinal epithelial cells and their engraftment have not been examined in detail. This is particularly critical, in light of the fact that the inner lining of the human gastrointestinal tract is replete with epithelial cells that undergo constant renewal with rapid turnover, resulting in exfoliation into the fecal stream of up to a hundred billion intestinal epithelial cells every 24 hours in a human subject.[10] Furthermore, the intestinal mucosa located under the epithelial cell lining maintains its integrity and steady state of continuously generating clusters of rapidly proliferating progenitor cells present in the lower third mucosal crypts under physiological conditions. The rate of cellular replication is balanced against the rate of cellular terminal differentiation and exfoliation into the fecal stream.[11–13] Previous work has demonstrated that exfoliated colonic epithelial cells can be isolated from human stool and remain viable for a week at room temperature.[12,14,15] Thus, viable exfoliated colonocytes in donor stool have the potential to engraft and persist in the recipient's colonic mucosa.

We have postulated that the clinical efficacy of FMT may involve many more steps than simple intraluminal transfer and engraftment of various microbes to the recipients gut because of the difficulty of microbial epithelial adhesion in the intestinal lumen. Failure of probiotics in many of these clinical settings has been attributed to poor microbial adhesion to intestinal mucosa. In vitro studies on isolated exfoliated colonic epithelial cells from human fecal samples have demonstrated active secretion of cell adhesion molecules, such as e-cadherins which can potentially enhance their ability to anchor to the recipient's colonic mucosa. We have postulated that FMT may be associated with allogenic engraftment of these exfoliated cells in the donor stool.[11] To study this cellular engraftment, we decided to test the presence of male Y chromosome (ChrY) and the sex-determining region Y (SRY) gene DNA in fecal samples of female FMT recipients with male donors. The presence of ChrY in fecal samples has been examined to identify the sex of a particular species in veterinary medicine, forensic medicine, as well as in social and evolutionary anthropology.[16,17]

Materials and Methods

Experimental Strategy

To determine the engraftment of cells from a normal donor subject to a recipient Clostridium difficile infection (CDI) patient, we chose to use stool samples paired between a male donor and a female recipient in which the male ChrY and SRY gene are followed over time following the FMT procedure. An increase of the male ChrY signal in the female stool over a measurement period from 1 week to 24 months after FMT was used to evaluate potential engraftment of the donor cells in the recipient.

RCDI Patients and Donors

Donors and patients with recurrent CDI selected for the FMT procedure were carefully screened according to criteria established previously. The patients went through a preliminary physical examination followed by cessation of antibiotic therapy for at least 48 hours. Established bowel preparation procedures for colonoscopy were followed. Stool samples were collected from donors and patients before FMT and processed as previously described including storage of aliquots in a stool bank at-80 °C. FMT was performed as described earlier. Post-FMT stool samples were collected from patients during follow-up visits which spanned from 1 week to 24 months following the procedure.

Next-Generation Sequencing (NGS) Assay for Total and ChrY Human DNA

Approximately 1.8 mL of frozen whole stool samples from donors and patients were sent to Admera Health, LLC (South Plainfield, NJ) for DNA extraction and library preparation. In brief, the genomic DNA (gDNA) was extracted using the NucleoSpin Soil kit (MACHEREY-NAGEL) and the concentration of gDNA was assessed by the Qubit double stranded DNA High Sensitivity DNA assay kit (Thermo Fisher). Libraries were prepared with the Nextera XT DNA Library Preparation Kit (Illumina) following the manufacturer's protocol. The concentration of final libraries was measured by quantitative polymerase chain reaction (qPCR) and equal amounts of DNA libraries were pooled, denatured with 0.2N NaOH, and then run on the NovaSeq S4, generating approximately 40M 150-nt paired-end reads per sample.

Paired-end FASTQ files were uploaded to Galaxy (usegalaxy.org, Afgan 2018) and analyzed using a custom bioinformatics pipeline. The trimmomatic tool was used to trim 10 bases from the ends of each read followed by genomic alignment using Bowtie2 against the hg38 reference human genome. A filtering step was used to select alignments in which reads are mapped in a proper pair and with a MAPQ quality score of 20 or greater. Idxstats was used to tabulate counts of each chromosome and featureCounts was used to tabulate SRY gene counts.

Digital PCR Assay for SRY Gene Copy Number

Total gDNA was isolated from stool samples using the QIAamp Fast DNA Stool mini kit (Qiagen cat# 51604) per the

manufacturer's instructions. Absolute copy numbers of SRY and Ribonuclease P genes were quantified using TaqMan SRY copy number assay (FAM dye) (Thermo Fisher #4400291) and TaqMan RNaseP copy number reference assay (VIC dye) (Thermo Fisher #4403326)) on the Naica digital polymerase chain reaction (PCR) system. Twenty five microliter reactions utilizing 5 uL of stool gDNA, 1X Perfecta Multiplex ToughMix, 1 uM Fluorescein, and 1X of each primer/probe mix were loaded on Sapphire chips and run on the Naica Geode at 95C for 10 min followed by 45 cycles of 95C for 30 sec, and 60C for 15 sec. The Sapphire chip crystals were scanned using the Naica Prime3 and analyzed using Crystal Miner software.

Q-PCR Assay for the SRY Gene Signal

Total gDNA was isolated from stool samples using the QIAamp Fast DNA Stool mini kit (Qiagen cat# 51604) per the manufacturer's instructions. Lysis conditions in this protocol are optimized to increase the ratio of human DNA to nonhuman DNA; however, nonhuman DNA is not excluded. gDNA was heated to 95C for 10 min to melt strands and increase primer per probe accessibility and then placed on ice. Relative human ChrY copy numbers were quantified by qPCR using an SRY gene primer/probe and an RNaseP copy number control primer/probe (TaqPath ProAmp Master Mix (Thermo Fisher #A30866), TaqMan SRY copy number assay Hs01026408_cn (Thermo Fisher #4400291), TaqMan RNaseP copy number reference assay (Thermo Fisher #4403326)). Reactions utilized 2 uL of stool gDNA per 10 uL reaction and were run at 95C for 10 min followed by 45 cycles of 95C for 15 sec and 60C for 60 sec on the StepOnePlus Real-Time PCR System. delta cycle thresholds were calculated as the difference between the cycle threshold (CT) of SRY and the CT of RNaseP.

Results

Our study population consisted of 52 subjects: 30 FMT donors (18 female and 12 male) and 22 FMT recipients (18 female and 4 male) (Table). Of the female FMT recipients, 12 had a male donor and 6 had a female donor. Of the male FMT recipients, 1 had a male donor and 3 had female donors. Stool samples were obtained from FMT recipients before and after FMT and stool samples from multiple time points were collected from some subjects. Post-FMT samples were collected at median time point of 4 months (IQR 1–5) after FMT administration. In total, 89 stool samples were collected and analyzed.

To estimate the human chromosomal content of total stool gDNA, we used an NGS data analysis pipeline to quantify the percentage of reads mapping to the human genome and the percentage of reads mapping to ChrY in the 89 stool gDNA samples. We also quantified SRY gene read counts, along with SRY gene abundance by both qPCR and droplet digital polymerase chain reaction (ddPCR) in 45 fecal samples from 15 donors (12 males and 3 females) and 15 CDI patients (13 females and 2 males) before and after FMT. We were able to detect SRY gene signal in 7 of 11 male fecal samples by ddPCR and in none of 22 female fecal samples. SRY gene detection by qPCR was less sensitive, being positive in only 3 of the 11 male samples. To reduce sampling error, we excluded from %ChrY analysis 38 samples that had less than 12,000 total human chromosomal read counts. We set this minimum threshold based on where an analysis of male and female donor control samples showed the expected ChrY abundance levels.

The percentage of ChrY read counts out of total human chromosomal DNA counts was quantified as %ChrY for each sample. As expected, the male donor samples had significantly higher %ChrY content than female donor samples (0.293% vs 0.0109%, $P < .0001$) (Figure 1). Surprisingly, 27 of 31 female-only samples had nonzero ChrY read counts. The sensitivity of ChrY detection in fecal samples was determined to be 90% in our study and specificity was 97%.

Post-FMT samples from females that received FMT from a male donor had higher %ChrY than all female-only samples including donors and recipients (0.0213% vs 0.00986%, $P = .03$) (Figure 1). Additionally, the change in %ChrY from before to after FMT was higher for female recipients with a male donor compared to a female donor (0.0146% vs 0.00406%, $P = .34$, ns); however, this failed to reach statistical significance (Figure 2A).

Four female recipients with a male donor showed a large %ChrY increase >0.02%, while no such large increase was observed with a female donor (Figure 2B, Figure A1E). Change in %ChrY did not strongly correlate with %human

Table. Total Number of Samples is Shown by Subject Sex for Donors, Pre-FMT Recipients, and Post-FMT Recipients. For Some Subjects, Samples From Multiple Time Points Were Analyzed. For %ChrY Analysis, Only Samples With >12,000 Human Reads Were Included to Reduce Sampling Error, Mean, and SD Are Shown. This Minimum Cutoff Was Determined Based on Analysis of Male and Female Control Samples

	Male			Female			
Number of samples	Donor	Pre-FMT	Post-FMT	Donor	Pre-FMT	Post-FMT (female donor)	Post-FMT (male donor)
N (total)	14	4	7	20	18	11	18
N (>12,000 human reads)	4	2	3	11	13	7	12
NGS %ChrY (SD)	0.293% (0.081)	0.149% (0.187)	0.260% (0.0480)	0.0109 (0.00695)	0.00852% (0.00750)	0.0106% (0.00792)	0.0213% (0.0264)
		0.251% (0.0979)			0.00986% (0.00724)		

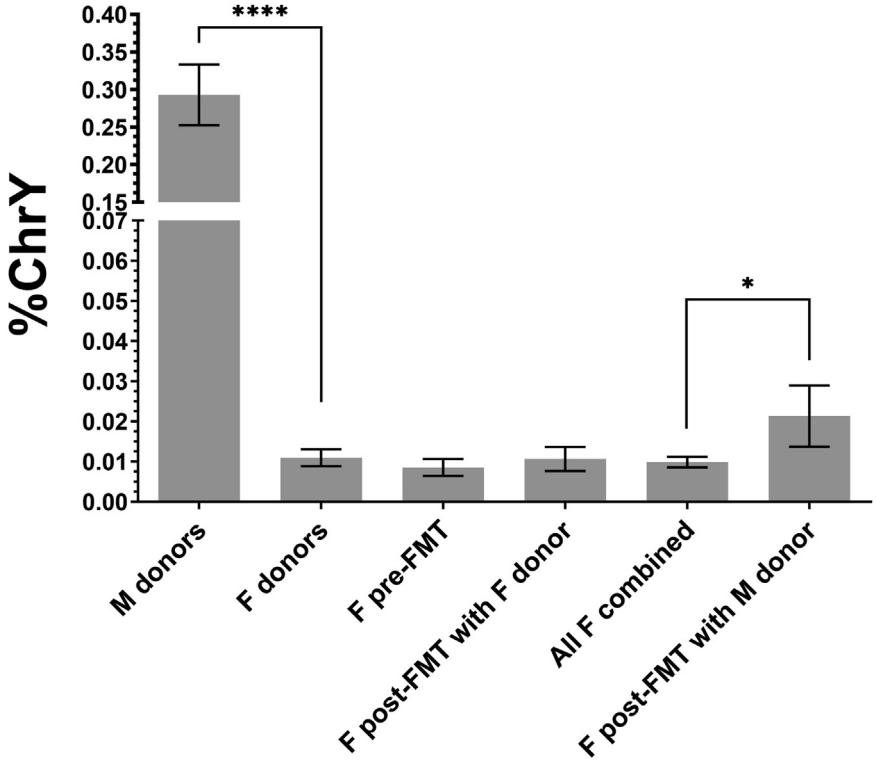

Figure 1. NGS-based %ChrY for donor, pre-FMT, and post-FMT samples. Samples with <12,000 human NGS reads were excluded from the analysis, mean and SD shown.

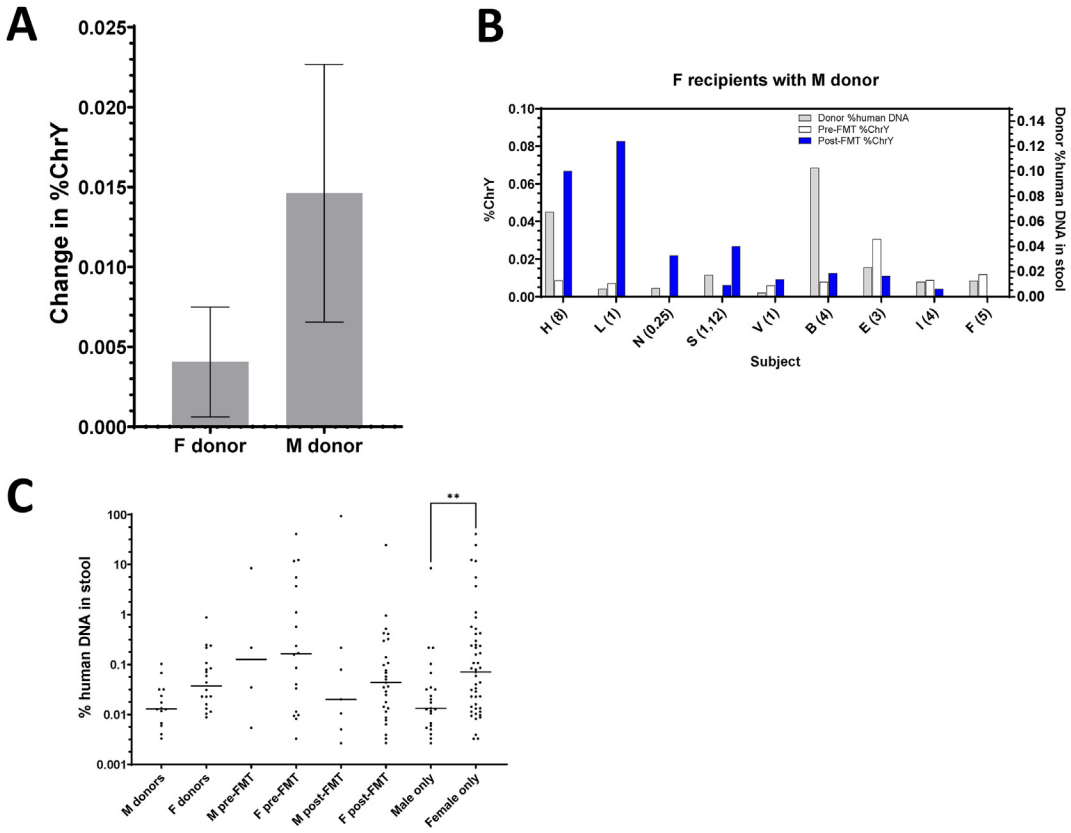

Figure 2. (A) Aggregated change in stool %ChrY pre-FMT and post-FMT for female recipients with a female donor or a male donor, mean and standard error of mean shown. (B) Individual donor %human DNA in stool (right axis) and pre-FMT and post-FMT %ChrY by NGS for female recipients with a male donor (left axis). Values in parenthesis indicate post-FMT follow-up time point in months. Missing bars indicate zero ChrY reads detected. (C) Percentage of human DNA in stool samples for male vs female donors and recipients, median shown.

DNA in the donor's stool, indicating other factors may play a role in cellular engraftment efficiency. Interestingly, female stool samples had significantly more human DNA compared to males (0.071% vs 0.013%, $P = .0087$) (Figure 2C); additionally, the percentage of human DNA in the stool of female donors was significantly correlated with age of the donor ($P = .038$), while no such correlation was found with male donors (Figure A1C-D).

Discussion

In the last 30 years, the biological role of ChrY has emerged as a principal determinant of gonadal sex in humans and other mammalian species, and it has been demonstrated that mammalian embryos with ChrY develop testes, while those with X-chromosome develop ovaries.[18] Furthermore, the presence of the SRY gene on the ChrY is primarily responsible for male phenotype. The method used in these studies included PCR based detection of the SRY gene present on ChrY.[19] Furthermore, detection of ChrY by qPCR in cell-free fetal DNA in maternal blood is commonly used for noninvasive fetal sex determination. In one such study, a 96% positive detection rate of a male fetus was reported with maternal plasma.[20]

Among healthy donors, we observed a percentage of human DNA in stool similar to previous estimates. However, among pre-FMT patients, we observed higher and more varied levels of human DNA, with 1 sample harboring up to 41% human DNA in stool (Figure 2C). This may be due to the antibiotic-induced depletion of microbes along with increased exfoliation of colonocytes and blood loss from the inflamed mucosal surface. Following FMT, the levels of human DNA in stool returned closer to healthy donor levels. In addition, we observed that among donors, pre-FMT, and post-FMT subjects, females consistently harbored higher amounts of human DNA in their stool compared to males, presumably due to higher prevalence of colitis in this group of patients.

Using shotgun NGS analysis applied to extracted human DNA from fecal samples of healthy donors as well as patients with RCDI, we were able to quantify the percent total human DNA and ChrY in each sample. Among healthy donors, the percentage of human DNA in stool was similar to the previous publications on this subject.[21]

Presence of ChrY was significantly higher in fecal samples collected from healthy male donors as compared to female donors. This difference was striking and was in line with previously reported observations in sika deer from Japan.[16] This group of investigators reported correct gender identification by a PCR essay in 34 of the 37 samples. Failure to achieve a perfect gender identification has been attributed to PCR inhibitors in stool samples. We are not aware of other human studies examining the presence of ChrY in fecal samples collected from healthy and diseased human cohorts. Using 0.1% rise in ChrY signal with greater than 12,000 reads, we have observed the sensitivity of 90% of ChrY detection in male donor fecal samples. The specificity of 97% was noted in this group of patients which included male and female donors and RCDI patients. Similarly, ChrY detection in human fecal samples has been proposed in isolated cases in forensic medicine.[17]

Interestingly, a significant increase in ChrY was seen in female recipients of FMT from male donors (Figure 1). Four of the 9 females who received FMT from male donors showed a strong ChrY signal at variable time intervals extending up to several weeks to months after FMT administration. We were able to detect SRY gene in 7 of 11 male fecal samples by ddPCR but none in the female fecal samples. Failure to detect ChrY signal in the other 5 of the 9 female recipients may be attributed to PCR inhibitors in stool samples and variable engraftment efficiency. These observations suggest that cellular engraftment from male stool samples administered to the females may have occurred in addition to microbial engraftment as previously reported in this group of patients.[3]

In this study, we sought to characterize chimerism in human stool DNA by analysis of the relative abundance of ChrY. Such analysis has great difficulty due to the small amount of human DNA present in stool compared to a large excess of microbial DNA. In line with the finding in this study, it has been previously estimated that DNA from colonic cells sloughed from the colonic mucosa represents 0.01%–0.1% of total stool DNA.[21] Furthermore, DNA originating from potential donor-derived engrafted cells would represent an even smaller fraction compared to host-derived DNA from exfoliated colonocytes. Other possible explanations for detection of ChrY could be a dietary source of male mammalian DNA such as mammalian meat consumption (beef, ham, lamb, etc) or oral transmission from an intimate male partner. Previous human and animal dietary studies using fecal DNA metabarcoding have shown that dietary DNA can be detected and quantified by PCR and metagenomic sequencing methods.[22]

These findings suggest that the donor-derived exfoliated intestinal epithelial cells in fecal samples may engraft in the colonic mucosa of FMT recipients. These engrafted donor epithelial cells may be responsible for altering the gut microenvironment and facilitate the engraftment of various microbial elements of the stool in patients with RCDI, bacterial dysbiosis and other GI disorders. Further investigation is needed to assess the mechanisms of cellular and microbial engraftment, and the role donor-derived cells may play in the long-term outcome of FMT in human intestines.

Supplementary Materials

Material associated with this article can be found in the online version at https://doi.org/10.1016/j.gastha.2024.10.008.

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Authors' Contributions:
Padmanabhan P. Nair: Conceptualization, writing – original draft. Sudhir K. Dutta: Conceptualization, methodology, investigation, project management, funding acquisition, writing – original draft. Elad Firnberg: Methodology, investigation, visualization, writing – original draft. Sandeep Verma: Investigation, writing – original draft. Laila Phillips: Methodology, investigation.

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Ethical statement:
This study was reviewed and approved by the institutional review board of Sinai Hospital of Baltimore; IRB number 1826. Written informed consent was obtained from all study subjects, including both patients and patient-selected healthy donors.

Data Transparency Statement:
All relevant data are within the manuscript. FASTQ raw data files related to this study have been uploaded to the NCBI Sequence Read Archive (BioProject ID PRJNA705895).

Reporting Guidelines:
Helsinki Declaration.