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Research Article

Gut Microbiota Composition and Its Association with Hydroxyurea Response in Beta Thalassemia Major Patients

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Abstract

Hydroxyurea (HU), a fetal hemoglobin (HbF) inducer, effectively alleviates symptoms in beta-thalassemia patients; however, its efficacy varies among individuals, potentially due to differences in HU metabolism and gut microbiota composition. Previous research has suggested that both factors can significantly impact drug metabolism and disease progression, including in thalassemia. This study aimed to establish a link between the gut microbiota and HU response in beta-thalassemia major patients. Stool samples were collected from a total of 45 beta-thalassemia patients aged 5-20 years and classified into three groups with 15 patients in each group: responders, non-responders, and those not using HU-based therapy on the basis of transfusion requirement. Notably, HU users tested negative for the Xmn-1 mutation. Sequencing was performed on the V3-V4 hyper-variable region of the 16S rRNA gene. The analysis uncovered noteworthy distinctions in the gut microbiota among the three groups at both the genus and species levels. The response to HU was associated with butyrate-producing bacteria from the phylum *Firmicutes*. Responders exhibited an enrichment of butyrate-producing bacteria such as *Faecalibacterium*, *Butyrivibrio*, *Oscillobacter*, *Gemmiger*, and *Eubacterium*. Non-responders, on the other hand, had higher levels of *Prevotella*, *Mitsuokella*, and *Treponema*. Non-users were characterized by an abundance of *Succinivibrio*, followed by *Bacteroides* and *Megasphaera*. These findings suggest that altered gut microbiota may contribute to inter-individual variation in HU response, highlighting specific microbes that could potentially serve as biomarkers for thalassemia or predict HU efficacy.

Keywords: Beta-Thalassemia, Hydroxyurea, Gut microbiota, Hydroxyurea responders, Hydroxyurea-non-responders, 16S rRNA Sequencing

1. Introduction

Inter-individual variation in response to a drug is the key factor contributing to treatment failure and drug toxicity. Various studies have highlighted the involvement of the human gut microbiome in drug metabolism, variability, and efficacy. Certain drugs, such as Proton pump inhibitors (PPI), antibiotics, metformin, statins, beta-blockers, antidepressants, opiates, paracetamol, oral contraceptives, and many more, have been shown to alter the composition and function of the gut microbiome (Weersma, Zhernakova, and Fu 2020).

Hydroxyurea, known for inducing fetal haemoglobin and addressing the α/β globin imbalance to enhance erythropoiesis,

(Cappellini et al. 2018), is an FDA-approved treatment for sickle cell disease and widely used for managing beta-thalassemia. Extensive evidence supports its positive impact on hematological parameters (Hb, HbF, MCV, MCH) and transfusion requirements in thalassemia patients, as demonstrated by a large body of evidence. However, the drug's effects remain variable (Ansari et al. 2011, Banan 2013, Musallam et al. 2013). Limited studies suggest that hydroxyurea metabolism plays a role in the diverse drug responses observed (Yahouédéhou et al. 2018, Walker et al. 2011).

Additionally, it has been demonstrated that the composition of the gut microbiota can impact hydroxyurea metabolism. Previous research indicates that hydroxyurea increases the abundance of the *Firmicute* phylum and its beneficial butyrate-producing species, while also exhibiting toxicity towards certain bacteria (Delgadinho and Ginete 2022, Eickhardt-Dalbøge and Ingham 2022, Yang et al. 2022). This dual effect on gut microbiota results in an enrichment of beneficial bacteria and elimination of pathogens.

The human gut microbiome, housing trillions of bacterial species, plays a crucial role in various physiological processes (Valdes et al. 2018). Dysbiosis, characterized by alterations in composition and function of gut microbiota, is associated with several diseases, including those affecting the cardiovascular (atherosclerosis, hypertension), gastrointestinal (Inflammatory bowel disease, irritable bowel syndrome, celiac disease), Endocrine (Diabetes, obesity, hyperthyroidism), Respiratory (Allergies, Asthma, and central nervous systems (Epilepsy, autism, depression) in which one or more beneficial gut microbial species are reduced. This alteration has adverse effects on health and provides an opportunity for the growth of pathobionts whose metabolites negatively impact the host, contributing to the disease pathophysiology (Lynch and Pedersen 2016, Gomaa 2020, Jackson et al. 2018). Existing literature also indicates dysbiosis in hemoglobinopathies, such as beta-thalassemia (Alizade et al. 2017, Alsaluki, Abd Alshibly, and Hassen 2017, Lun et al. 2021) and sickle cell disease (Brim et al. 2021, Lim et al. 2018). However, limited studies have explored the role of the gut microbiome in beta-thalassemia, revealing an abundance of gram-negative bacteria, particularly *Escherichia. coli*, in the gut microbiota of beta-thalassemia patients (Alizade et al. 2017, Alsaluki, Abd Alshibly, and Hassen 2017, Lun et al. 2021).

Beta-thalassemia is an autosomal recessive disorder characterized by ineffective erythropoiesis, resulting in anemia that necessitates regular blood transfusions for affected individuals. The combination of ineffective erythropoiesis and frequent transfusions leads to iron overload, causing toxicity in various organs; hence, the primary treatment approach focuses on managing this overload through a combination of blood transfusion and iron-chelation therapy (Origa 2017). The prevalent carrier rate of Beta-Thalassemia is 1.5% globally. In Pakistan, around 5000-9000 children are born with β-thalassemia every year. The carrier rate is around 5-7% with 9.8 million carriers in the total population. The exact carrier rate is lacking in Pakistan (Ansari et al. 2019). Despite the improvement in the quality of life for thalassemia patients through these interventions, achieving effective control remains challenging in our country. Management of beta-thalassemia poses a burden in underdeveloped countries like Pakistan, which have limited resources. Treatment is costly, burdens the patient financially, and is related to poor patient compliance. Hydroxyurea emerges as a cost-effective medication that has proven beneficial for many

Table 1. Baseline characteristics of participants

	HU responders	HU non-responders	HU non-users	p-value
Gender n(%) **Males** **Females**	11(73%) 4 (26%)	10 (66.6%) 5 (33%)	9 (60%) 6 (40%)	0.745
Age (years) n(%) **5-9** **10-14** **15-19**	15 (100%) 0 0	7 (46%) 5 (33%) 3 (20%)	4 (26% 8 (53%) 3 (20%)	0.0023*
BMI for age (percentile)	14.80	14.70	14.90	0.845
Blood transfusion per year	0	12	22	<0.0001*
Iron chelator Deferasirox n(%)	2 (13.3%)	12 (80%)	11 (73.3%)	0.0008*

*n= number of participants

individuals with beta-thalassemia (Ansari et al. 2018). The approach to alter the gut microbiota centers around enhancing beneficial members or reducing harmful pathogens to address various diseases. Techniques involving antibiotics, dietary adjustments, and the utilization of probiotics and prebiotics have shown promising results (Young 2017). While hydroxyurea is widely used in beta-thalassemia, its interaction with gut microbiota and the potential impact on treatment efficacy have not been investigated. Exploring the role of gut microbiota in beta-thalassemia patients treated with hydroxyurea could open new avenues for optimizing treatment strategies, improving outcomes, and reducing variability in response.

Research increasingly suggests that gut microbiota plays a significant role in drug metabolism, immune regulation, and inflammation, all of which can influence treatment outcomes. The microbiota may affect the metabolism of hydroxyurea and modify the inflammatory and oxidative stress environment that impacts erythropoiesis, potentially altering the response to treatment. Investigating the potential association between hydroxyurea response variation and the gut microbiome could offer valuable insights into modifying the microbiome to enhance beta-thalassemia treatment outcomes. This study aims to investigate whether gut microbiota composition is associated with the response to hydroxyurea in beta-thalassemia patients.

2. Methods & Materials

2.1. Study Design

This cross-sectional study, approved by the institutional review board at Dow University of Health Sciences (IRB-1989/DUHS/Approval/2021/1045), took place from May 2021 to December 2022. It involved 45 beta-thalassemia major patients aged 5-20, recruited from the Children's Hospital, Karachi, and Omair Sana Foundation. Sample size was determined using PASS software based on the methodology used by (Villanueva-Millán et al. 2017).

Written informed consent was obtained from all participants and parents of the participants who were underage. Participants were screened for eligibility and divided into three groups: Hydroxyurea Responders were patients on

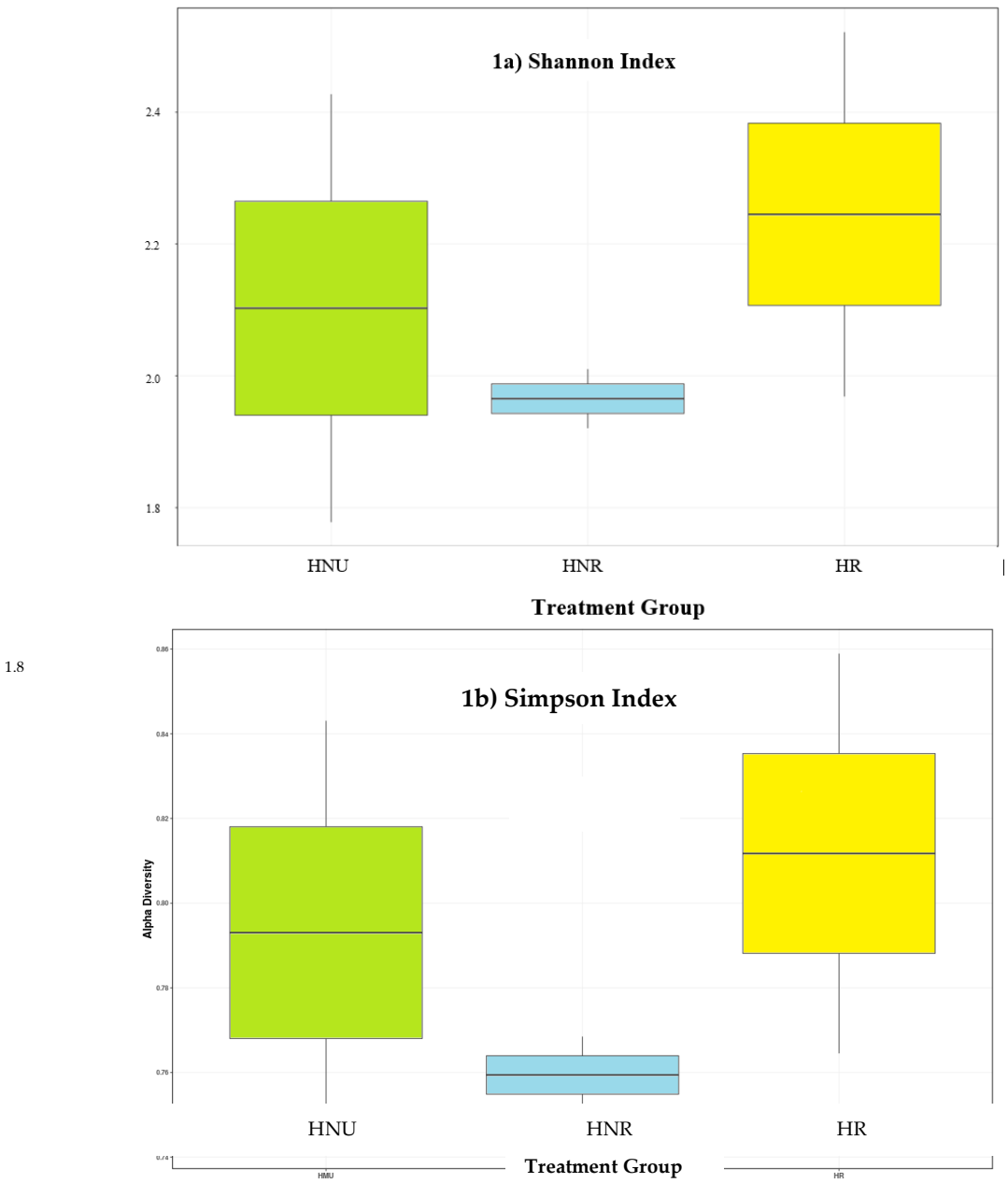

Figure 1. Alpha diversity box plots comparing the responders, non-responders, and non-users of HU groups for (a) Shannon index (p-value= 0.9%), and (b) Simpson Index (p-value= 0.9%).

hydroxyurea for at least 6 months, who either no longer needed transfusions or had over a 50% reduction in transfusion requirements. Hydroxyurea Non-responders were patients on hydroxyurea for at least 6 months who experienced less than a 50% reduction in transfusion needs. Non-users were patients not taking hydroxyurea.

2.2. DNA Extraction and 16S rRNA Sequencing

Patients were instructed to collect a stool sample, no more than 4 hours old, which was collected from their homes and stored at -80°C at the molecular pathology section of Dow Diagnostic Research & Reference Laboratory (DDRRL) till further processing. DNA extraction was done

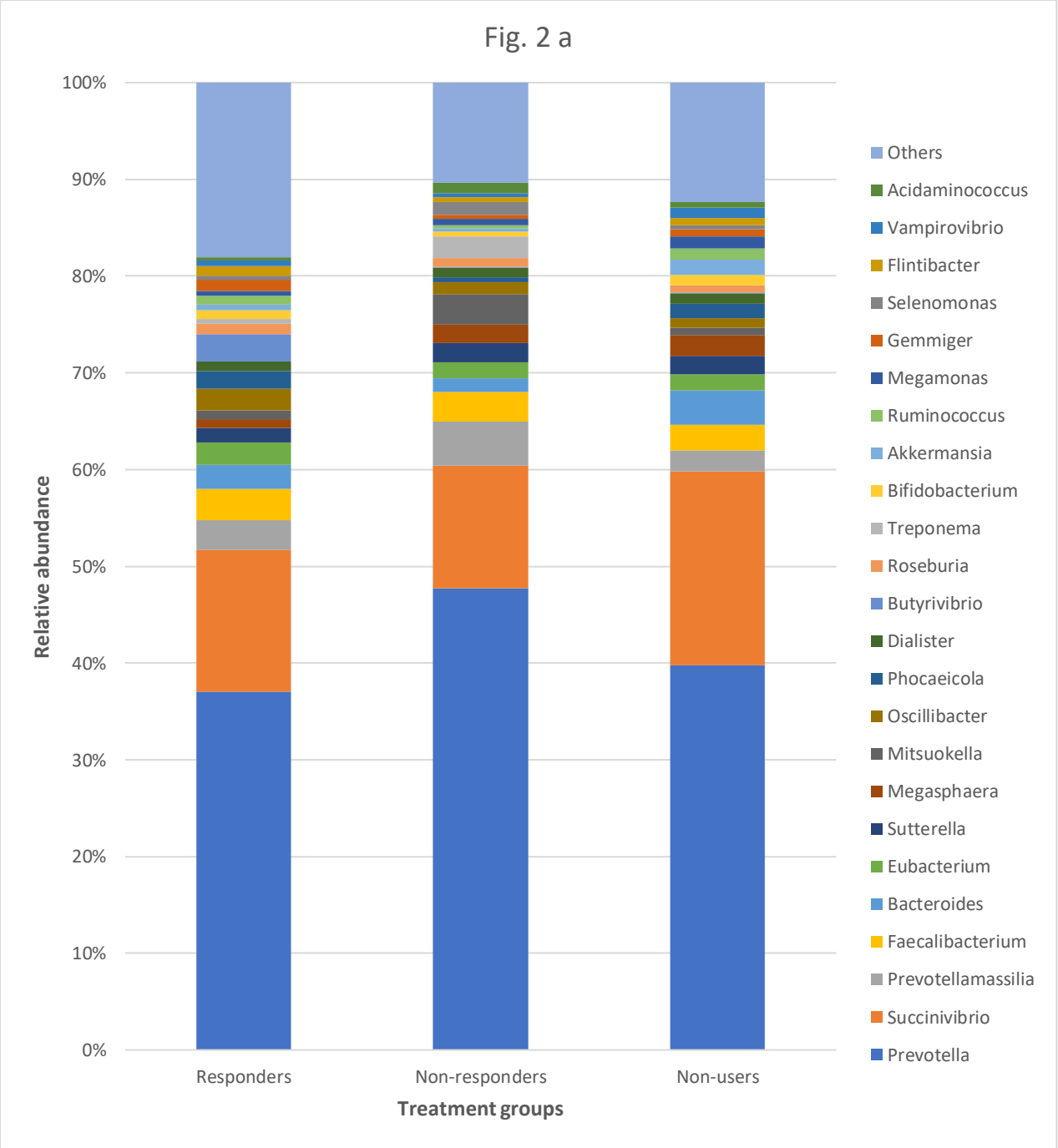
Fig. 2 a
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative abundance
Responders
Non-responders
Non-users
Treatment groups
Others
Acidaminococcus
Vampirovibrio
Flintibacter
Selenomonas
Gemmiger
Megamonas
Ruminococcus
Akkermansia
Bifidobacterium
Treponema
Roseburia
Butyrivibrio
Dialister
Phocaeicola
Oscillibacter
Mitsuokella
Megasphaera
Sutterella
Eubacterium
Bacteroides
Faecalibacterium
Prevotellamassilia
Succinivibrio
Prevotella

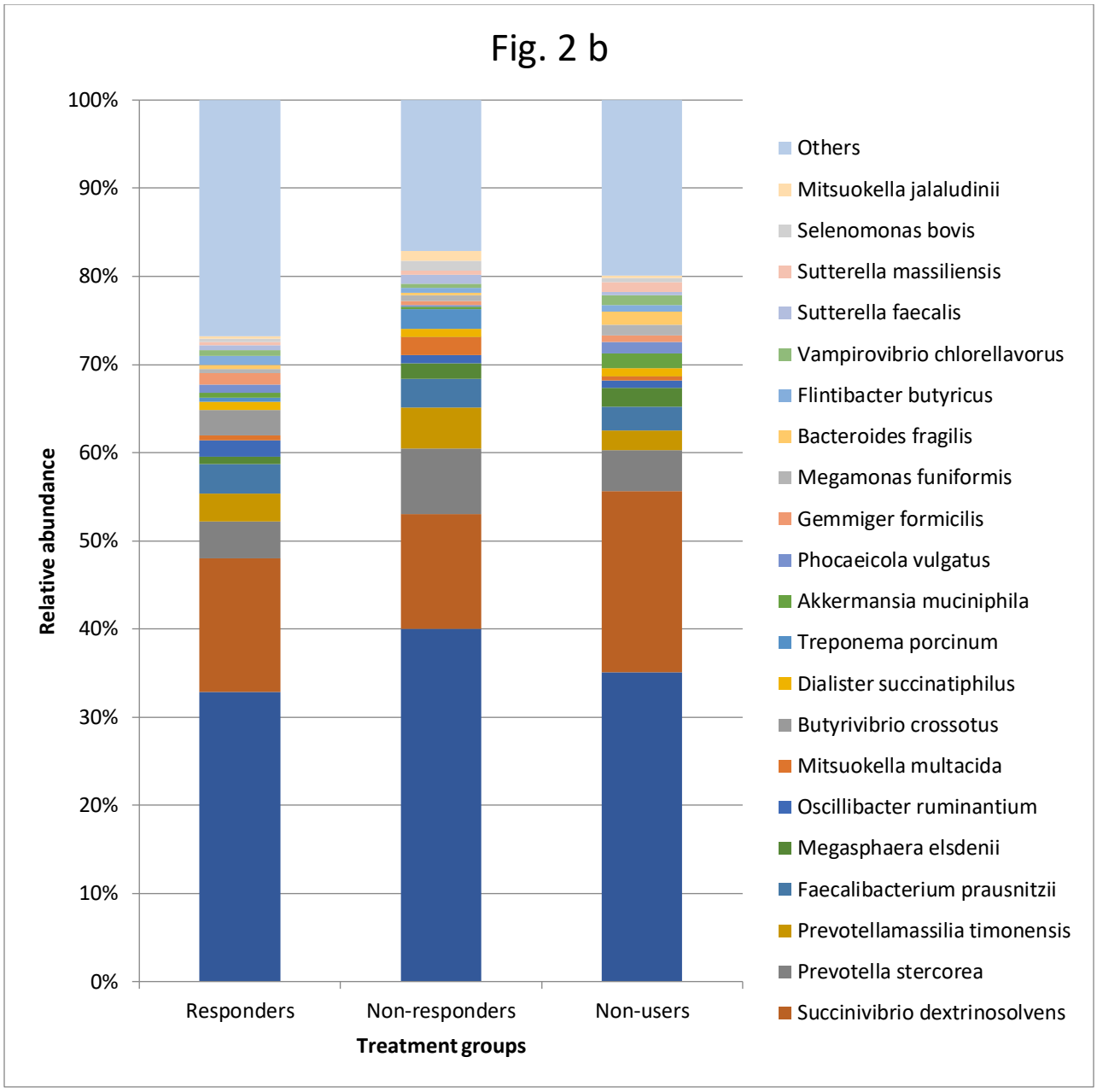

Figure 2. Microbiome profile in hydroxyurea responders compared with hydroxyurea non-responders, and non-users in beta-thalassemia major. (a) Genus p-value=0.0042 (b) Species p-value <0.000*

using QIAamp DNA Stool Mini Kit (QIAGEN, Germany) as per manufacturer's instructions (QIAGEN. 2020). DNA purity and concentration were measured spectrophotometrically (Thermo Scientific, USA) and stored at -20°C until further processing. Genomic DNA was sent to Macrogen Inc, Korea) for PCR of V3-V4 hypervariable region and 16S rRNA sequencing. Preparation of the library was done following 16S Metagenomics sequencing library preparation protocols by Illumina with a primer set 341F: CCTACGGGNGGCWGCAG and 805R: GACTACHVGGGTATCTAATCC. The

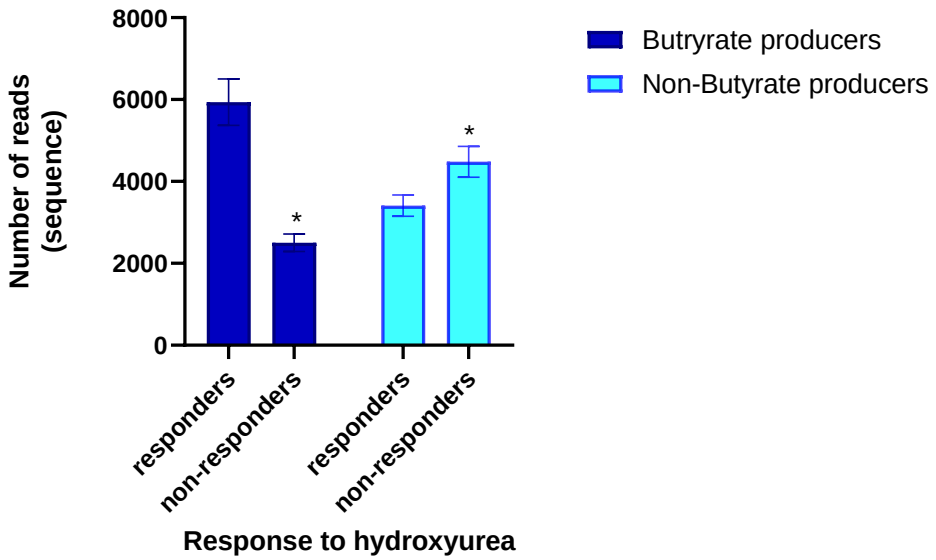

Figure 3. Bar graph representing the association by chi-square test between hydroxyurea response and butyrate producers of the Phyla *Firmicutes*. *p*-value <0.01*

sequences obtained were compared with the reference database for taxonomic classification.

2.3. Data Processing

NGS data was received in the form of FASTq format. It was trimmed and filtered using PRINSEQ. Pair-end reads were merged with PANDAseq. The sequences were aligned with reference sequences of NCBI BLAST. MEGAN software was used for taxonomic analysis. R package (v3.2.0) was used to compute alpha diversity indices such as the Simpson index and Shannon index (alpha diversity).

2.4. Statistical Analysis

All data were statistically analyzed using Graph Pad Prism V. 9.4.1. Normally distributed data were expressed as mean + S.D. Non-parametric data were expressed as median and quartiles. For the three-group comparison, the Kruskal-Wallis test was applied with Dunn's multiple comparison test for inter-group comparison. For the association between the response of hydroxyurea and the abundance of butyrate producers, a chi-square test was used. Alpha diversity was calculated using the R package. The significance level was set at $\alpha = 0.05$.

3. Results

3.1. Patient Characteristics

Most study participants were males (66%) and aged 5-12 (73%). BMI-for-age percentiles, based on WHO standards, (Organization 2007) categorized 71% of participants as having a healthy weight, while 29% were underweight. Most common ethnic backgrounds included Balochi (20%), Sindhi (20%), and Urdu-speaking (17%). Among hydroxyurea responders, the median annual transfusion rate six months' post-therapy was zero (IQR: 0-5), while non-responders had a median of 12 transfusions per year (IQR: 12-20). The majority of participants used the iron-chelator Deferasirox (53.3%), primarily among non-responders (73.3%) and non-users (73.3%), while the majority of responders did not require iron-chelation therapy. Frequencies are shown in Table 1

3.2. Gut Microbiota Diversity

We did not observe significant differences between the microbiota diversity of the responders, non-responders, and non-users of HU (Shannon: p=0.9, Simpson: p=0.9). Fig.1

3.3. Comparison of Gut Microbiota

No significant differences were found in gut microbiota composition among hydroxyurea responders, non-responders, and non-users at the phylum level (p-value >0.05), except at the genus ($p = 0.0042$) and species levels ($p < 0.0001$). The predominant phyla across all groups were *Bacteroidetes* and *Firmicutes*, with lower abundance of *Proteobacteria. Prevotella* and *Succinivibrio* were the most common genera. In responders, *Butyrivibrio* and Oscillibacter were more abundant; in non-responders, Mitsuokella and *Treponema* were prevalent; and in non-users, *Succinivibrio, Bacteroides*, and *Megasphaera* were abundant (Figure 2).

The most abundant butyrate producers belonging to the phyla *Firmicutes* genera (>1%) were found to be associated with hydroxyurea response (p-value <0.01*), Figure 3. The gut of hydroxyurea responders shows enrichment in butyrate-producing bacteria. Genera with more than 1% relative abundance of the Phylum Firmicutes were analyzed for differences in hydroxyurea response. Butyrate producers include Faecalibacterium, Oscillobacter, Butyrivibrio, Eubacterium, Roseburia, and Ruminococcus. Non-butyrate producers include *Megasphaera, Mitsuokella, Dialister, Megamonas, Gemmiger, Selenomonas,* and *Accidaminococcus*. Overall, the responders showed higher levels of butyrate-producing *Firmicutes*, while non-responders had more *Bacteroidetes* species, and non-users had more *Proteobacteria* species.

4. Discussion

The gut microbiome composition is influenced by factors like race, age, health, disease, and drug usage (McCoubrey et al. 2022). While we did not observe significant differences in the overall microbiota diversity (alpha and beta diversity) among hydroxyurea responders, non-responders, and non-users, we did find distinct shifts in the microbial composition across the groups. These shifts were characterized by differences in the relative abundance of specific genera, such as increased butyrate-producing bacteria in hydroxyurea responders and a higher presence of *Prevotella* and *Succinivibrio* in non-responders and non-users, as shown in Table 1 and Figure 2. In our study, hydroxyurea responders demonstrated a marked enrichment of butyrate-producing genera within the *Firmicutes* phylum, such as *Faecalibacterium*, *Oscillobacter*, *Eubacterium*, and *Butyrivibrio*. These beneficial microbes are well-documented for their role in maintaining gut health by producing short-chain fatty acids (SCFAs), particularly butyrate, which supports colonocyte energy, enhances intestinal barrier integrity, and exhibits anti-inflammatory properties (Fig. 3) (Parada Venegas et al. 2019). These mechanisms are particularly critical in managing chronic conditions like thalassemia, where gut dysbiosis and inflammation are common (Visitchanakun et al. 2020). Key butyrate-producing genera within *Firmicutes* include *Faecalibacterium, Butyrivibrio, Eubacterium, Fusobacterium, Clostridium, Megasphaera, Roseburia, Dialister, Bifidobacterium,* and *Ruminococcus*. A decrease in butyrate-producing bacteria can foster an environment favoring the growth of pathobionts (Nagata et al. 2022)..

Our findings are consistent with studies in other conditions, such as inflammatory bowel disease (IBD), where enrichment of butyrate-producing bacteria through probiotic interventions has been shown to reduce systemic inflammation and improve intestinal repair (Chen and Vitetta 2020). Furthermore, previous research on hydroxyurea-treated sickle cell disease patients similarly highlighted an increase in beneficial *Firmicutes* species, reinforcing the association between

hydroxyurea use and gut microbial composition shifts (Delgadinho and Ginete 2022). Additionally, Studies in mice show that improving gut permeability with probiotics, such as *L. rhamnosus GG*, may reduce infections and improve therapy outcomes (Visitchanakun et al. 2021). These findings suggest that probiotics enhancing butyrate production could improve hydroxyurea response in non-responders, who exhibit lower levels of butyrate-producing bacteria, potentially leading to gut dysbiosis and reduced drug efficacy.

The increased presence of *Butyrivibrio* and *Eubacterium* in hydroxyurea responders is noteworthy because they induce fetal hemoglobin (HbF) production by inhibiting histone deacetylase, which is a key action of hydroxyurea. By enhancing the hydroxyurea's efficacy, they may provide relief for thalassemia patients (Shah and Dwivedi 2020).

Beyond beta-thalassemia, hydroxyurea has been shown to influence gut microbiota in various diseases. For instance, in polycythemia vera patients on hydroxyurea, *Bacteroides* levels were much higher (24%) than those observed in our study, while *Faecalibacterium* levels were comparable (4% vs. 3.25%) (Figure 2) (Eickhardt-Dalbøge and Ingham 2022). In atherosclerosis, hydroxyurea use decreased the abundance of *Lactobacillus, Escherichia. Coli* and *Helicobacter*, while increasing members of the *Lachnospiraceae* family, a pattern reflecting their broader microbial impact (Yang et al. 2022). Moreover, hydroxyurea is shown to be toxic to certain microbes, including *Escherichia. coli* (Davies et al. 2009). The relative abundance of *Prevotella*, along with *Escherichia. coli* was notably high in β-thalassemia minor pregnant women (Lun et al. 2021). Similar observations are apparent in studies on beta-thalassemia, indicating a high abundance of gram-negative bacteria, particularly *E. coli*, in the gut microbiota of beta-thalassemia patients (Alizade et al. 2017, Alsaluki, Abd Alshibly, and Hassen 2017). In contrast, our study revealed that *E. coli* abundance in all three groups was below 1%, potentially reflecting the beneficial effects of hydroxyurea on limiting harmful bacteria.

Our study highlights that the non-responders group (HNR) exhibited a significant enrichment of *Prevotella*, *Prevotellamassilia*, and their species (*P. copri, P. stercorea*, and *P. timonesis*) (Figure 2). *Prevotella* is a common gut commensal, particularly in agrarian societies, and is a major producer of propionate, an SCFA involved in physiological processes. However, it is also associated with inflammatory conditions like rheumatoid arthritis, periodontitis, HIV, and metabolic diseases (Iljazovic et al. 2021). The genera *Mitsuokella* (from the Firmicutes phylum) and *Treponema* (from the Spirochaetes phylum) were found exclusively in non-responders, with very low or negligible amounts present in the other two groups. *Treponema*, another short-chain fatty acid (SCFA) producer, aids in fiber breakdown and is commonly found in agricultural microbiomes (Precup and Vodnar 2019b). However, research on anti-diabetic responses has shown that both *Prevotella* and *Mitsuokella* produce trimethylamine n-oxide (TMAO), a compound linked to cardiovascular diseases (Tsai et al. 2022). Elevated TMAO levels and reduced SCFA production are indicators of gut dysbiosis (Tsai et al. 2022, Precup and Vodnar 2019b) a pattern observed in thalassemia-related gut alterations. For instance, studies on thalassemic mouse models have highlighted colonic inflammation and significant shifts in microbial metabolites, mirroring the dysbiosis noted in non-responders (Sriwichaiin et al. 2022).

In hydroxyurea (HU) non-users, the gut microbiota was characterized by a higher

prevalence of the phylum *Proteobacteria*, specifically the genus *Succinivibrio* and its species *Succinivibrio dextrosolvens* (Figure2). An increased presence of Proteobacteria is often linked to gut dysbiosis and inflammation, which are commonly observed in metabolic disorders (Shin, Whon, and Bae 2015). Although *Succinivibrio* is a short-chain fatty acid (SCFA) producer, common in agrarian communities (Precup and Vodnar 2019a), it is also associated with various health conditions, such as coronary heart disease and Crohn's disease (Liu et al. 2020, Markandey et al. 2022). In our study, we also observed that *Bacteroides* levels were higher in hydroxyurea non-users compared to hydroxyurea users. These commensal bacteria support gut barrier integrity and produce SCFAs that prevent dysfunction but can also have adverse effects, contributing to conditions like diarrhea, colorectal cancer, and inflammatory bowel disease. *Bacteroides fragilis*, for example, promotes immune regulation yet may damage the intestinal barrier through toxin release (Wang et al. 2021). Similarly, the genus *Megasphaera*, which was prevalent in non-users, functions as an SCFA producer in humans, although its role in human health is less understood (Bhute et al. 2016).

The presence of beneficial bacteria that boost SCFA production can help reinforce the gut barrier, potentially improving dysbiosis and offering therapeutic benefits.

5. Conclusions

This study highlights several beneficial bacterial species that can serve as therapeutic biomarkers for beta-thalassemia and hydroxyurea efficacy, or be used as probiotics in treatment management. Understanding how hydroxyurea metabolism and the physiological changes associated with thalassemia influence the gut microbiome, along with identifying microbial species linked to improved treatment responses, could help optimize global treatment strategies.

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Ethics Approval and Consent to Participate

This cross-sectional study was approved by the institutional review board at Dow University of Health Sciences (IRB-1989/DUHS/Approval/2021/1045). Written informed consent was obtained from all patients. When, below 16 years of age, consent was taken from their parents.

Availability of Data and Materials

Dow University of Health Sciences has ownership rights to the data and material of the current study. The Institute has permitted the corresponding author to make the data set available upon reasonable request.

Declaration of Competing Interests

The authors declare that they have no competing interests associated with this study.

Authors' Contributions

Ayesha Khan: conceptualization, investigation, methodology, visualization, and writing the manuscript. Saeed Khan: Supervision, resources, methodology, and project administration. Saqib H. Ansari: Supervision, investigation, and resources. Ayaz Ahmed: Supervision, resources, and formal analysis. Asif I. Khan: Software. Muniza Omair: Formal analysis.

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