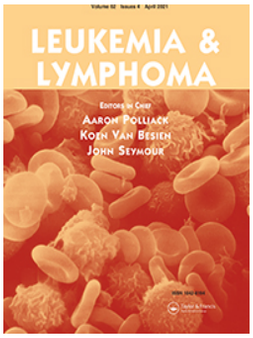

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ORIGINAL ARTICLE

Investigating the gut microbial community and genes in children with differing levels of change in serum asparaginase activity during pegaspargase treatment for acute lymphoblastic leukemia

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ABSTRACT

Asparaginase (ASNase) is an effective treatment of pediatric acute lymphoblastic leukemia (ALL). Changes in ASNase activity may lead to suboptimal treatment and poorer outcomes. The gut microbiome produces metabolites that could impact ASNase therapy, however, remains uninvestigated. We examined gut-microbial community and microbial-ASNase and asparagine synthetase (ASNS) genes using 16SrRNA and metagenomic sequence data from stool samples of pediatric ALL patients. Comparing ASNase activity between consecutive ASNase-doses, we found microbial communities differed between decreased- and increased-activity samples. *Escherichia* predominated in the decreased-activity community while *Bacteroides* and *Streptococcus* predominated in the increased-activity community. In addition microbial ASNS was significantly (p=.004) negatively correlated with change in serum ASNase activity. These preliminary findings suggest microbial communities prior to treatment could affect serum ASNase levels, although the mechanism is unknown. Replication in an independent cohort is needed, and future research on manipulation of these communities and genes could prove useful in optimizing ASNase therapy.

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Acute lymphoblastic leukemia; asparaginase treatment; microbiome; Bayesian model; pediatric

Introduction

Asparagine synthetase (ASNS) converts aspartate into asparagine. Asparagine, is used in DNA and protein synthesis, and depletion leads to cell death [1–3]. While most human cells contain ASNS, lymphoblastic leukemia cells have no or low levels of this enzyme [3–5]. The lack of ASNS in those cells makes them dependent on extracellular sources of asparagine. L-Asparaginase (ASNase) converts asparagine to aspartate and at sustained high levels this enzyme depletes circulating asparagine, leading to leukemic cell death [1–3,6]. This dependency has led to the use of ASNase as an important therapy in treatment of pediatric acute lymphoblastic leukemia (ALL) [3,6].

ASNase used in treatment is derived from bacteria and a pegylated form of *Escherichia coli* ASNase (PEGASNase) is used by most patients. Individuals that exhibit hypersensitivity to PEGASNase are switched to *Erwinia*-ASNase [6]. ASNase-related complications include side effects (thrombosis, pancreatitis) and clinical hypersensitivity reactions. Patients can develop anti-ASNase antibodies that trigger hypersensitivity reactions, and can cause the enzyme to be less effective, decreasing asparagine depletion and leading to poorer patient outcomes. Some patients develop levels of ASNase insufficient to deplete asparagine but without side effects, 'silent inactivation', which undetected leads to suboptimal treatment and poorer patient outcomes. Antibody presence in patients treated with native-ASNase ranges from 28 to 96% [6–15]. Antibodies in patients treated with PEGASNase are lower ranging from 2 to 29% [6,13,15,16]. There is uncertainty however in these numbers as many studies include patients switched from

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Supplemental data for this article can be accessed here.

native-ASNase to PEGASNase [6,12–16]. In addition an inverse relationship between ASNase activity and antibody optical density suggests antibodies may result in partial inactivation [14].

ASNase treatment efficacy relies on significant depletion of circulating asparagine for sustained periods of time. Therapeutic monitoring is critical to ensure sufficient levels of ASNase activity to maintain asparagine depletion. Monitoring levels of asparagine directly in the blood is problematic due to technical limitations [17]. Instead, serum ASNase activity is monitored as a proxy for asparagine levels, with ASNase inversely related to asparagine. Current consensus is the ASNase activity target trough should be $\geq$0.1 IU/mL. The predictors of serum levels of ASNase activity and mechanisms leading to antibody formation are not clear but altered exposure to microbiome-derived ASNase or the counter-enzyme ASNS is an underexplored avenue.

The gut microbiome contains trillions of bacteria that produce metabolites necessary for health [18], including microbial taxa that produce short chain fatty acids, bile acids, choline, and B-vitamins, in addition to other metabolites in the gut environment [19–21]. Such metabolites can reach the blood of the host, thereby directly impacting host metabolite availability [22]. Visconti et al. [23] examined healthy individuals and found 86% of gut microbiome metabolic pathways, and 34% of gut microbiome species, were associated with metabolites identified in the blood. Critically, the microbiome may be altered by development of cancer and, reciprocally, cancer progression and course of treatment could be altered by changes in the microbiome [24].

In this study, we investigate the relationship between the composition of the gut microbiome and microbial ASNase and ASNS genes and changes in serum ASNase levels during treatment in pediatric ALL patients. ASNase treatment involves delivery of a series of 2–9 doses of PEGASNase, with higher risk patients receiving more doses. To investigate the relationship between the microbiome and change in serum ASNase levels, we recorded serum levels in pediatric ALL patients and sampled their gut microbiomes between consecutive doses. We observed cases of both increasing and decreasing serum ASNase levels, and investigated community structure of a patient's gut microbiome between these two clinical outcomes. We further examined the distribution of, and taxa contributing to, microbial ASNase (ansA and ansB) and ASNS genes to determine their association with observed changes in patient serum levels.

Methods

We investigated 12 pediatric ALL patients (Table 1) undergoing care (IWK Health Centre) for which serum ASNase levels were measured for two consecutive doses of PEGASNase and a stool sample was collected between doses. Seventeen stool samples fit these criteria. Serum ASNase level was measured 5–13 days after PEGASNase treatment. The difference in consecutive measurements was recorded (Table 1), and

Table 1. Demographic and clinical characteristics of acute lymphoblastic leukemia (ALL) patients included in this study.

Patient ID	Age (years)	Gender	Induction therapy	Dose number	Stool to dose (days[a])	Serum change (IU/mL)	Risk	Dose[d] (IU/m^2)	MRD[e]
SD1_P1	14.64	M	AALL1131	2	2	–0.066	H[b]	2500	–
SD2_P1	14.64	M	AALL1131	3	0	–0.7	H	2500	–
SD3_P2	7.74	M	AALL1131	4	65	–0.719	S[c]/H	2500	+
SD4_P3	0.93	F	Interfant-06	2	13	–0.002[f]	H	2500	–
SD5_P3	0.93	F	Interfant-06	3	28	–0.088	H	2500	–
SD6_P4	1.27	M	AALL0932	2	14	–0.424	S/H	2500	–
SI1_P4	1.27	M	AALL0932	3	25	0.05	S/H	2500	–
SI2_P5	11.74	M	AALL1131	4	33	0.703	H	2500	–
SI3_P6	3.97	F	AALL0932	2	106	0.12	S	2500	–
SI4_P7	10.03	M	AALL1131	7	10	0.53	H	2500	–
SI5_P7	10.03	M	AALL1131	4	17	0.088	H	2500	–
SI6_P8	4.62	M	AALL1131	2	45	0[f]	H	2500	–
SI7_P8	4.62	M	AALL1131	4	76	0.368	H	2500	–
SI8_P9	3.27	F	AALL0932	4	73	0.593	S/H	2500	+
SI9_P10	1.95	F	AALL0932	2	108	0.271	S	2500	–
SI10_P11	0.08	F	Interfant-06	2	103	0.355	H	1650	–
SI11_P12	0.79	F	Interfant-06	2	26	0.342	H	2500	–

[a]Days between stool collection and dose of PEGASNase.
[b]H: high risk ALL.
[c]S: standard risk ALL.
[d]Intravenous PEGASNase dose administered to pateints.
[e]MRD: minimal residual disease.
[f]Samples not included in analysis.

activity was considered decreased when serum ASNase levels declined from previous measurement. Serum ASNase activity levels were measured by CLIA certified Next Molecular Analytics Laboratory (Chester, VA) and St. Justine Hospital Laboratory (Montreal, Canada). Standard blood collection and sample handling protocols were followed. The IWK Health Centre Ethics Committee approved this study and all patients or their guardians provided informed consent.

Sample preparation and sequencing details for both 16S rRNA and Shotgun metagenomes are provided in Supmat. Sequencing followed a standardized protocol (Microbiome Helper workflow pipeline [25]). Paired-end reads were joined using VSEARCH [26] and deblur [27] was used to correct reads and obtain amplicon sequence variants (ASVs). ASVs account for sequencing error and yield an exact sequence that can be directly compared to other studies. Additional details of ASV quality control and processing are provided in Supmat. Alpha-(Shannon diversity) and beta-(weighted Unifrac) diversity were obtained using QIIME2 [28]. A Bayesian modeling framework (BioMiCo [29]), which takes ASVs as input, was employed to infer how microbial communities differ between patients having serum decrease (SD) and serum increase (SI). Training and testing of BioMiCo models is described in Supmat.

Shotgun metagenome sequence reads were annotated via HUMAnN2 [30] according to metacyc pathways and UniRef90 clusters [31]. We identified reads corresponding to L-asparaginase-I (ansA), a low affinity enzyme located in the cytoplasm, and L-asparaginase-II (ansB) a high affinity secreted enzyme (used for ASNase treatment) according to the UniRef90 annotation. For each sample, both the counts per million reads, and proposed distribution of contributing taxa, were determined for ansA, ansB, and ASNS. We tested SI versus SD using the Wilcoxon rank sum test, and logistic regression (variable selection: Lasso [32] with backward elimination) to examine bacterial genes (ASNS, ansA, and ansB) and covariates (age, gender, disease risk, dose number, time between doses, time between stool and second dose and previous serum ASNase value) with change in serum ASNase level.

Results

Number of PEGASNase doses administered to patients ranged from 2 to 7. Among the 17 stool samples collected between doses, two samples were excluded from further analysis, because the change (0 and −0.002 IU/mL) was less then the error level of the ASNase activity assay (<0.05 IU/mL). The remaining 15 samples were examined and we identified 10 SI cases and five SD cases (Table 1). Average increase in serum levels was 0.342 IU/mL (0.05–0.7) and average decrease was −0.399 IU/mL (–0.066 to −0.72). One patient experienced both SI and SD, with remaining either SI or SD. Average age was 5 years (0.1–14.6 years) with 5 under the age of 3 (mean age 1) and 7 over 3 (mean age 8) (Table 1). Average age was 6 years (0.9–14.6 years), for SD patients and 4 years (0.08–11.7 years) for SI patients. No significant difference in age was found between SI and SD patients (p=.6). Time between stool sample collection and dose administration varied across samples, with mean number of days at 44 [0–108 days]. Average number of days between stool and dose were significantly smaller (p=.03) in SD samples, but was not found to be correlated with change in serum ASNase levels (p=.271). Longer time periods between PEGASNase dose and serum ASNase measurement could result in decreased serum ASNase levels, and this could explain the changes in levels between doses. Therefore, we examined these time differences in relationship to SI and SD labels and change in serum ASNase activity. Mean difference in time between dose and measurements was 12 h for SD (–36 to 58 h) and 23 h for SI (–36 h to seven days) and did not differ significantly between groups (p=.84), and was not correlated with change in serum activity (p=.96).

Mean sequence count in the 15 samples was 25,653 (2473–65,481) and 426 unique ASVs were identified, with 210 in >1 sample. The number of species present and the composition of these species can characterize the bacterial community in a particular environment. This is commonly referred to as alpha diversity and can be measured using Shannon diversity index (SDI). SDI measures both the number of taxa (in this case ASVs) and richness of those taxa, with low values indicating less diversity and high values more diversity. In these samples, the average SDI was 3 (0.3–5.7). Given age effects diversity [33,34], we divided samples into two groups, under 3 (U3) and over 3 (O3). Nine samples were O3 with an average SDI of 4.1 (2.5–5.7) and six samples were U3 with average SDI of 1.3 (0.3–3.4). SDI was correlated with age at start of treatment (linear regression; R^2=0.43, p=.004), and differed significantly between O3 and U3 group (Wilcoxon; p=.004). SDI was not correlated with change in serum ASNase (linear regression; p=.5) nor differed between SI and SD groups (Wilcoxon; p=.4).

Beta diversity differs from alpha diversity in that it summarizes compositional differences *between* rather then within microbial communities. Weighted Unifrac

distance, which takes into account relative abundance of taxa and their phylogenetic relationships, was computed from 16S rRNA sequence data pairwise between samples and visualized using principal coordinate analysis. Samples with more similar microbial communities have smaller distances and will cluster closer together. Comparison of distances within and between groups can be performed (PERMANOVA) to examine if microbial communities differ. Beta diversity inferred from 16S rRNA sequence data for these samples revealed differences in the community composition associated with both age (p=.004, R^2=0.13) and change in serum ASNase levels (p=.016, R^2=0.108) (Figure 1). Interestingly, the effect of age and ASNase change on microbial community composition was largely orthogonal (Figure 1) suggesting compositional differences based on age were different from change based on ASNase activity.

Bayesian inference of community structure [29] applied to 16S rRNA ASVs identified five predominant species-assemblages (Figure 2(a)) accounting for 0.95 and 0.78 of the posterior probability (PP) of SD and SI cases, respectively, with a unique mixture in the structure for the SD and SI associated microbial communities. Fifty-two ASVs (PP >0.01) contributed to one or more of these assemblages. Since assemblages do not make equal contributions to the two communities, the relative contribution of each ASV to SD and SI must be weighted according to its membership in each community assemblage. Following weighting, 30 ASVs had PP >0.01 for either SD or SI communities; their structure is shown in the cytoscape [35] network (Figure 2(b)). Several ASVs were identified as predominant in either the SD or SI community, and made a large contribution to the difference between their structures. The largest contributing

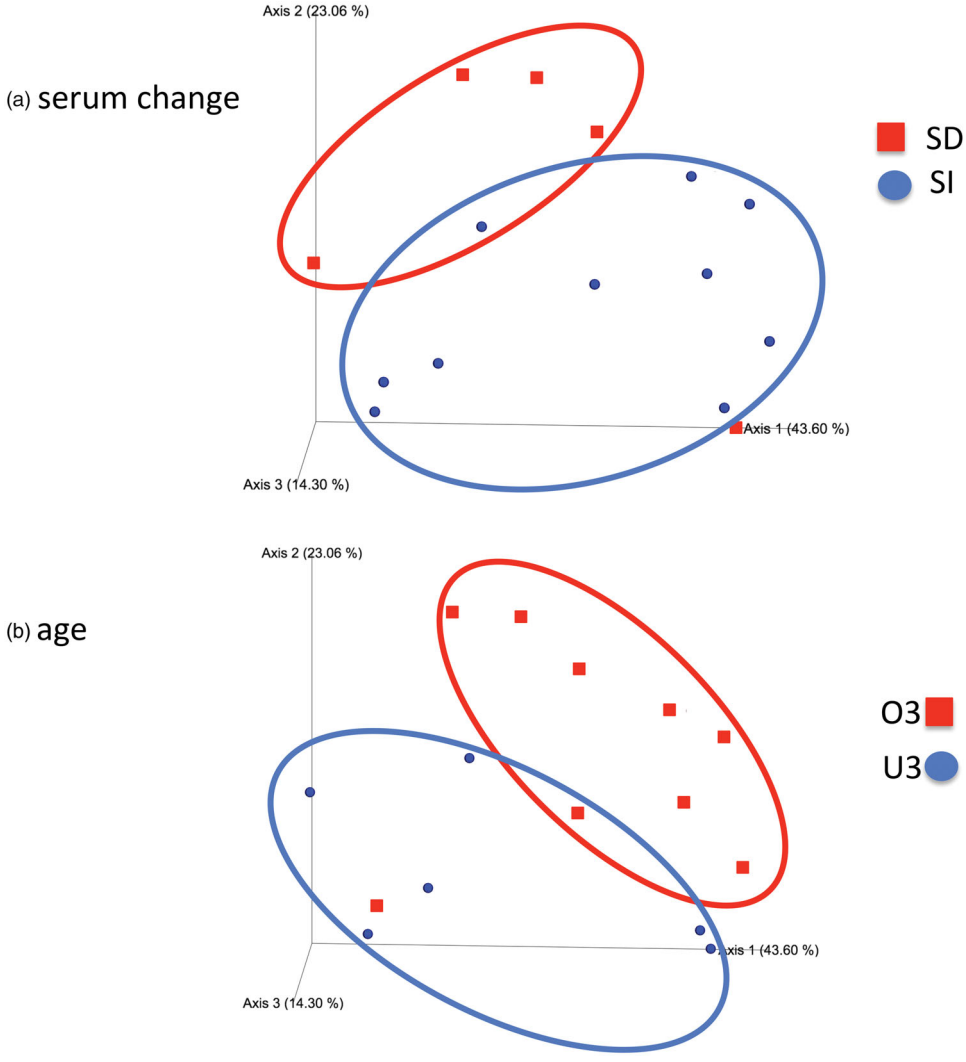

Figure 1. Beta diversity, shown as a Principal Coordinate Analysis ordination plot using the weighted Unifrac metric obtained from 16S rRNA sequences of stool samples from ALL patients. Each point represents a sample and the distance between points indicates the similarity of those points. (A) Samples are labeled by their serum change category: serum increase (SI) circles, serum decrease (SD) squares. (B) Samples are labeled by age group: over 3 (O3) squares, under 3 (U3) circles.

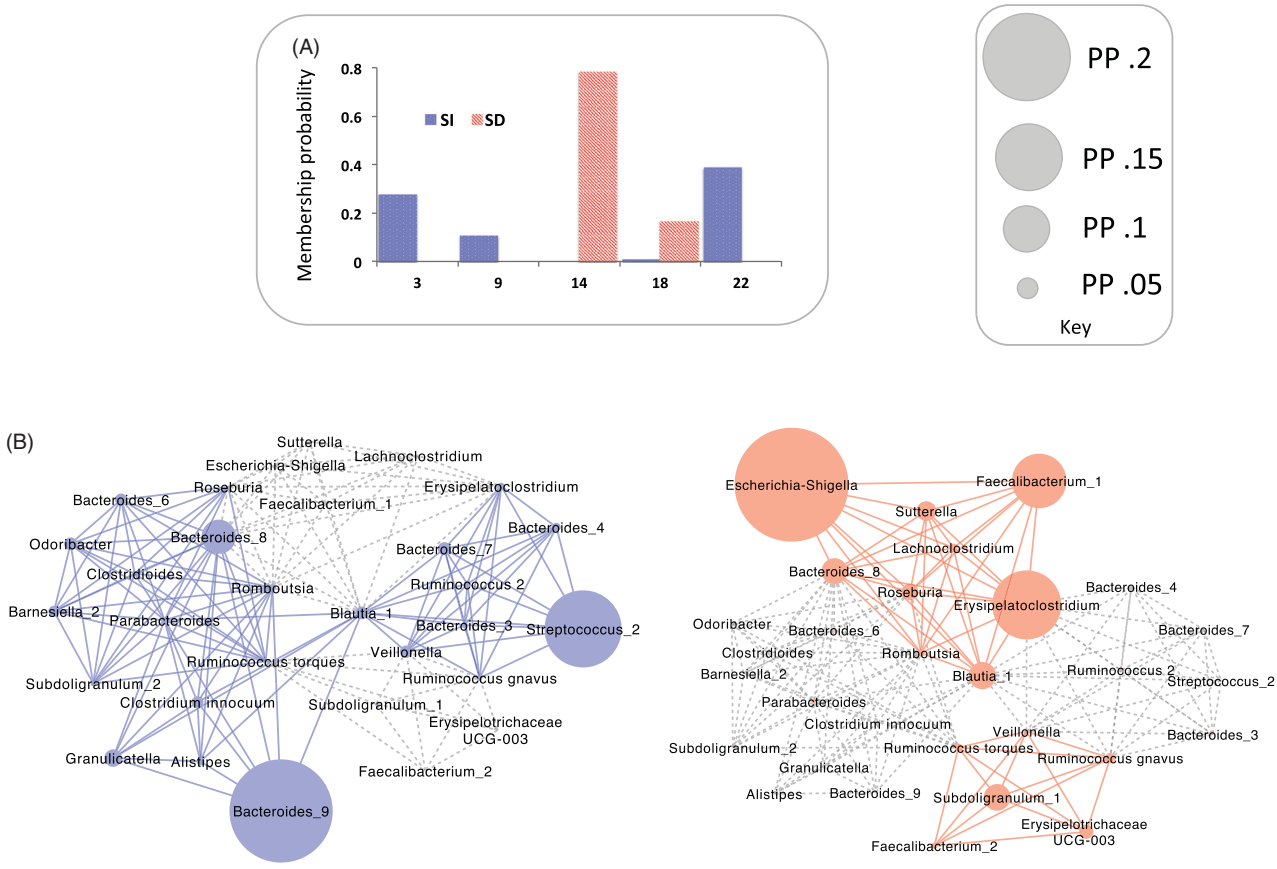

Figure 2. Community structure identified from BioMiCo analysis. (A) Posterior probabilities (mixture weights) for the predominant assemblages associated with the SI and SD gut communities. (B) Network of ASVs that comprise the SI- and SD-associated assemblages. Solid edges show connections between ASVs in SI predominate assemblages in the SI community and SD predominate assemblages in the SD community. Node sizes are the weighted-sum of the posterior probability of an ASV in the involved (SI or SD) assemblages. Only ASVs with weighted-sum of PP >0.01 in either SI- or SD-associated assemblages are shown.

ASV in the SD community was *Escherichia-Shigella*, with major contributions also from *Erysipelatoclostridium*, *Faecalibacterium*, *Subdoligranulum*, *Sutterella*, *Blautia*, and *Roseburia*. The predominant ASVs in the SI community were several lineages of *Bacteroides* and *Streptococcus*, as well as *Clostridium innocuum* and *Granulicatella*. Some ASVs were found in both groups (e.g. *Erysipelatoclostridium*, *Bacteroides*, *Blautia*, *Romboutsia*, and *Ruminococcus gnavus*) however at differing PP (Figure 2(b)). Using leave-one-out cross-validation [36] the trained model predicted direction of ASNase change in 73% of the replicates (AUROC = 0.75). Although prediction accuracy is promising, the limited number of samples requires further validation in additional datasets to determine extent to which the model has broad applicability and its ability to predict change in serum ASNase.

Functional capacity of the gut microbiomes was determined from metagenomic sequences. In particular, we identified counts of ASNS and ASNase genes, ansA and ansB, in the gut bacteria of 14 samples. Mean counts of ASNS (155, range 6–320) differed significantly (p=.007) between SI and SD samples (Figure 3(A)), and were negatively correlated with change in serum values (R^2=0.51, p=.004; Figure 3(B)). Mean counts of neither ASNase genes, ansA (165, range 63–331) or ansB (65, range 4–180), differed significantly between SI and SD (p=.52; p=.24 respectively; Figure 3(A)) and were not correlated with change in serum values (p=.84; p=.4 respectively; Figure 3(B)). We assessed linear regression models to examine the relationship between gene counts (ansA, ansB, and ASNS), age, gender, disease risk, days between stool collection and dose, days between doses, dose number, and serum ASNase level at previous dose, at predicting change in serum ASNase levels. Model selection using lasso and backward elimination identified ASNS counts (p=.001), dose number (p=.005), age at diagnosis (p=.006), previous serum ASNase levels (p=.02), and ansA counts (p=.09), in a model to

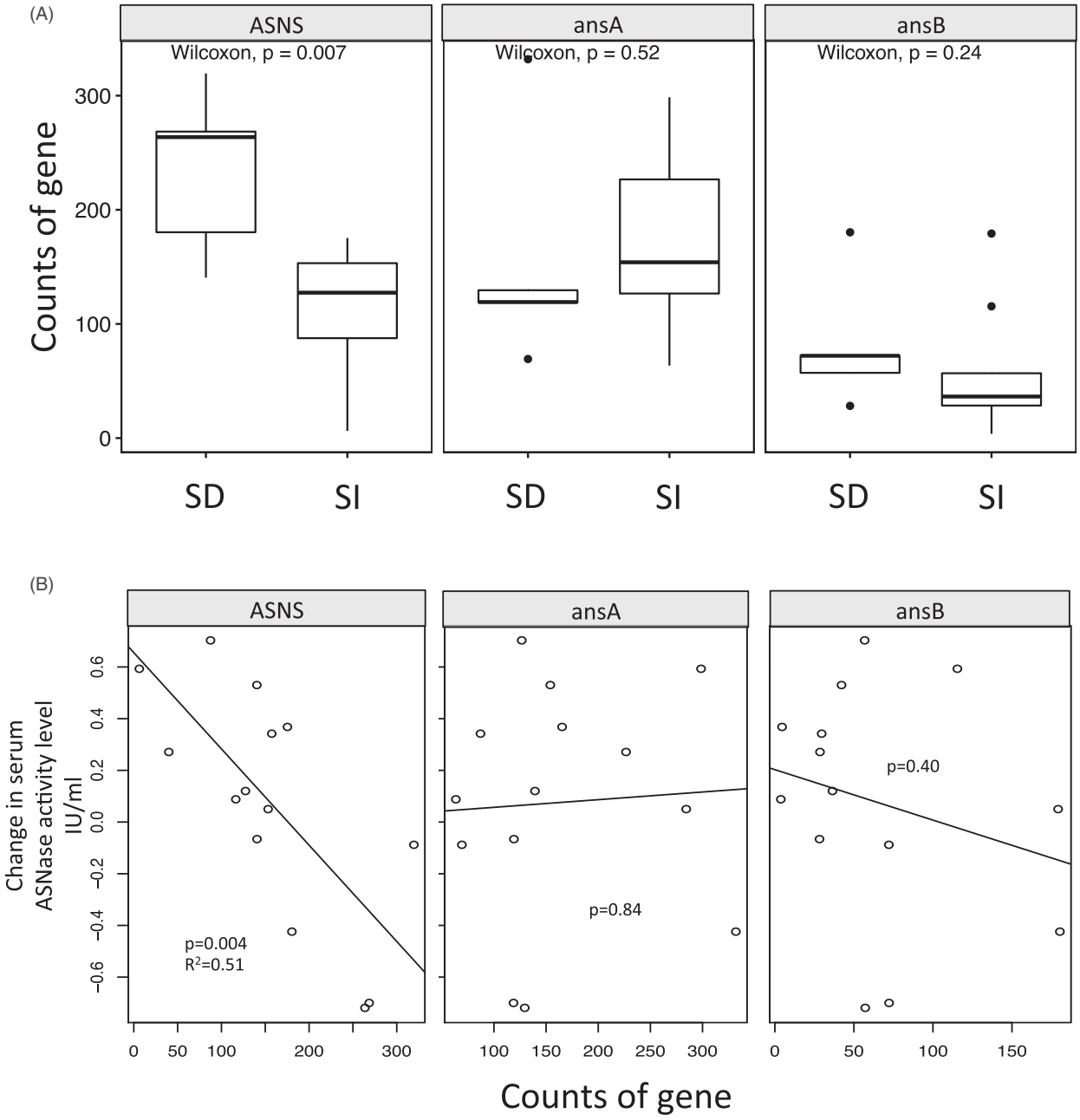

Figure 3. Counts of microbial asparagine synthetase (ASNS), L-asparaginase-I (ansA), and L-asparaginase-II (ansB) genes identified in stool samples of pediatric ALL patients compared by change in serum ASNase activity level. (A) Box plot of counts of ASNS, ansA, and ansB by patients with decreasing serum ASNase activity between measured doses (SD) and those that did not decrease between doses (SI). (B) Plots of counts of ASNS, ansA, and ansB genes in samples against change in serum ASNase activity level between doses. The *p* value shown is from the *F*-statistic using the gene counts to model change in serum ASNase activity level.

predict serum ASNase change (R^2=0.82, p=.001). All but dose number were negatively correlated with serum ASNase change.

We used homology-based methods [30] to assign taxonomic status to sequences of ASNS, ansA, and ansB genes. We identified 83 microbial species containing ASNS, 57 in >1 sample (Supplemental Table), 15 in >30% of samples, and only three in ≥50% of samples (Table 2). We identified 85 microbial species with ansA gene, 57 in >1 sample (Supplemental Table), 13 in > 30% of the samples, and four in ≥50% of samples (Table 2). We identified 30 microbial species with ansB gene, 22 in >1 sample (Supplemental Table), six in >30%, and two in ≥50% of samples (Table 2).

Examining the distribution of these genes, we find 12 taxa contain ansB but not ansA, while 46 contain ansA without ansB (Supplemental Table) with four of those identified in ≥50% of the samples (*F. prausnitzii*,

Table 2. Taxa identified in stool samples of ALL patients containing asparagine synthetase, L-asparaginase-I, and L-asparaginase-II genes in at least 30% of samples.

Taxa identified	ASNS	ansA	ansB
Actinobacteria			
Bifidobacterium longum		++	
Collinsella aerofaciens		+	
Bacteroidetes			
Alistipes putredinis		+	
Bacteroides ovatus	+	+	
Bacteroides stercoris	+	+	
Bacteroides uniformis	+	+	
Bacteroides vulgatus	+		+
Parabacteroides merdae	+	+	
Firmicutes			
Blautia [Ruminococcus] gnavus	++		++
Blautia [Ruminococcus] obeum	+		
Blautia [Ruminococcus] torques	+		
Clostridium symbiosum	+	+	
Clostridium ramosum	+		+
Clostridium bartlettii			+
Faecalibacterium prausnitzii	++	++	
Lachnospiraceae bacterium 7_1_58FAA	+		
Roseburia intestinalis	++	++	
Streptococcus sp.		+	
Streptococcus thermophilus		++	
Veillonella parvula			+
Proteobacteria			
Escherichia coli	+	+	++

(+) Taxa present in >30% of samples. (++) Taxa present in ≥50% of samples.
ASNS: asparagine synthetase; ansA: L-aspraginase-I; ansB: L-aspraginase-II.

R. intestinalis, *S. thermophilus*, and *Bifidobacterium longum*). Three taxa contained both ASNS and ansB without ansA (*B. vulgatus*, *R. gnavus*, and *C. ramosum*), and 36 taxa contained ASNS and ansA without ansB (Supplemental Table). Only six taxa (*Achromobacter piechaudii*, *B. fragilis*, *B. ovatus*, *B. thetaiotaomicron*, *Eubacterium biforme*, and *E. coli*) contained all three genes.

Taxonomic attribution of ASNS and ASNase genes permits assessment of lineage-specific contributions of genes to changes in serum levels. No taxa were correlated with serum change in lineage-specific analysis of ansB. Lineage-specific contribution of ansA and ASNS genes found *Faecalibacterium* (ansA: R^2=0.39, p=.017; ASNS: R^2=0.42, p=.012), and *Roseburia* (ansA: R^2=0.285, p=.049; ASNS: R^2=0.3, p=.04) were negatively correlated with change in ASNSase level.

Discussion

We examined the change in serum ASNase levels (SD and SI) between consecutive doses of PEGASNase and how the microbiome community differed prior to the second dose. While high inter-patient variability in ASNase activity is known [6,37] intra-patient variability has not been examined. Using Bayesian methods, we found community structure differed, with SD-community dominated by *Escherichia-Shigella*, *Faecalibacterium*, *Erysipelatoclostridium*, *Subdoligranulum*, *Blautia*, and *Roseburia*, and SI-community predominated by *Bacteroides,* along with *Streptococcus*, *Granulicatella*, and *C. innocuum*. Community differences were also manifested in bacterial genes related to asparagine, finding ansA and ASNS encoded by *Roseburia* and *Faecalibacterium* negatively correlated with serum ASNase levels.

The large contribution of *Escherichia* in the SD community is intriguing as *it* is the bacterial source of ASNase in PEGASNase. Whether this contribution triggers antibodies, possibly leading to enzyme inactivation [38] requires further investigation and could not be addressed in this current study. Future studies should examine change in ASNase levels, microbiome, and anti-ASNase and anti-PEG antibodies, to examine if gastrointestinal *Escherichia* is related to antibody production. If such a relationship is identified individuals with increased *Escherichia* might benefit from *Escherichia* reduction prior to PEGASNase treatment. Although a novel approach that needs to be tested formally shifting the microbiome to impact treatment has been used previously in Crohn's disease to success [39,40]. Improving our understanding of what impacts levels of serum ASNase is important, as suboptimal levels are associated with poor clinical outcomes resulting in higher risk of ALL relapse [41–43].

This is the first study to examine microbial ASNase (ansA and ansB) and ASNS genes in the microbial community present in ALL patients undergoing PEGASNase therapy. Many bacteria taxa contain low affinity cytoplasmic ansA (L-asparaginase-I), while fewer taxa contain the high affinity secreted ansB (L-asparaginase-II), the source of PEGASNase therapy. Databases such as KEGG do not differentiate between these genes grouping them (K01424) making examination of these genes difficult. Using UniRef annotation [31], we examined counts as they relate to the specific genes (ansA and ansB). We found taxa containing ansB genes to be less then half the number with ansA gene, and species with only ansA gene 4 times more likely then only ansB. Given the source of ASNase is the ansB gene, we hypothesized that its counts would be related to change in serum ASNase levels. This was not the case at either total counts or at individual taxa. While for ansA, the low affinity enzyme we had not expected a relationship with serum change, which was confirmed with total counts, but when combined with other factors was marginally significant (p=.09) in modeling ASNase change. Additional sampling is necessary to confirm these relationships. More studies examining ansA and ansB distribution in bacterial taxa

and annotating databases to reflect these differences are needed.

ASNS, produces asparagine, that has the opposite effect of ASNase. Lorenzi et al. [44] found that in some solid tumors ASNS expression levels were inversely correlated with ASNase activity. We found that counts of ASNS in the gut microbiome were significantly negatively correlated (p=.004, R^2=0.51) with change in serum ASNase levels; larger counts of ASNS gene were present when there was a larger decrease in serum ASNase activity. We note that gene count does not indicate expression level in this study, but is rather the metabolic potential present in the microbiome. Expression of ASNS however is regulated by amino acid deprivation and cell stress, increases in expression are seen upon asparagine deprivation [45]. While PEGASNase does not directly affect the intestine, it is not known whether decreased asparagine in the blood, due to PEGASNase, drives the transport of asparagine from the intestine to the blood. A deprived asparagine state in the gut could possibly lead to increased ASNS expression in the gut microbial community. Finally, recent studies have shown levels of human ASNS are negatively correlated with survival in a number of cancers [46]. While there is no direct mechanism for variation in ASNS levels to cause variation in serum ASNase levels there does appear to be correlation between these measures. Further independent mechanistic and confirmatory studies will need to be done to determine whether this relationship is reproducible and what mechanisms are involved. This highlights the requirement for further research into the role of bacterial ASNS during ASNase treatment.

One limitation of this study was the low number of available samples that met the eligibility criteria. Although we found significant differences between SD and SI samples, additional sampling is needed to confirm the differences found in the observed microbial communities. In addition, sampling over a larger more complete age distribution will allow assessment of age on both the regression models of serum ASNase change and the role of age on microbiome composition in ALL patients. Sampling across a larger range of doses will allow assessment of repeated doses on serum ASNase change. Finally, changes in the microbiome can occur quickly in response to changes in diet, treatment and environment [47–50]. To get the best snapshot of the microbiome prior to treatment samples should ideally be collected in the days prior to treatment. This was not the case for these samples as there was considerable variation in collection time (Table 1). Although number of days between stool collection and dose was included in the model predicting change in serum ASNase activity, this variation could not be accounted for in the Bayesian model. SD stool samples were closer to the second ASNase dose then SI samples which had much more variation (Table 1). This variation might explain the larger number of ASVs identified in the SI community. Limiting comparisons in future sampling to the days prior to ASNase dose will be useful to examine what impact this variation has on the microbial community.

In conclusion, even with limited number of samples we found differences in microbial community prior to treatment in patients whose serum ASNase levels decreased compared to increased. This study is also the first to examine the distribution of bacterial ASNase and ASNS genes in pediatric ALL patients during PEGASNase treatment. These preliminary data suggest that increased amounts of bacterial ASNS genes present may be associated with a decrease in serum ASNase levels, although there may be added complexity from other covariates that needs to be examined further. While currently no mechanism exists that implicates ASNS enzyme in directly decreasing ASNase activity, this is not the first study to find that ASNS and ASNase activity are inversely correlated [14,45,51,52]. There is still much we do not understand about the role the microbiome may play in cancer development and therapy [53–56]. Future work should focus on larger and more diverse sets of samples collected from stool in the days prior to treatment in order to (i) further investigate and validate in an independent cohort SD and SI community-level properties and the role of covariates (e.g. age and dose number) and (ii) examine interplay between serum ASNase level, antibodies, and bacterial taxa and genes (ansA, ansB, ASNS). These future analyses could lead to a predictive model that could use these markers to screen patients before ASNase treatment aiding in predicting sub-optimal or decreasing ASNase levels prior to treatment. These models could be useful to guide reduction in certain taxa (e.g. *Escherichia*) pretreatment.

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Data availability statement

The 16S rRNA gene and metagenomic sequencing data used in this study are available under accession number PRJEB41463 at the European Nucleotide Archive.

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