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Analysis of blood immune biomarkers and fecal microbiome in subjects with and without malignant solid tumors: report from a feasibility study

Haoran Gao[2], Devansh Acharya[1,2], Eliot N. Haddad[3], Sarah S. Comstock[3], Rick Jorgensen[2], Muhammad Hamdan[1], Hussein Al-Ahmad[1], Brittani Thomas[1], Ushasree Chamarthy[1], Venu Gangur[2], Gordan Srkalovic[1,*]

[1]Herbert-Herman Cancer Center, E W Sparrow Hospital, Lansing, MI 48912, United States
[2]Food Allergy & Immunology Laboratory, Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824, United States
[3]Microbiome Laboratory, Department Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824, United States

ABSTRACT

Background and Objectives: Cytokines and other immune regulatory molecules are critical for mounting an effective immune response against cancer. The gastrointestinal (GI) microbiome plays a significant role in the pathogenesis of cancer and the response to immunotherapy. The central hypothesis guiding this project was that specific immune biomarkers and microbiome profiles will serve as predictors of effective *vs*. ineffective immunotherapy in patients with malignant diseases. This pilot feasibility study aims to establish baseline immune markers and microbiome profiles in subjects with newly diagnosed malignant solid tumors (n = 10), healthy subjects without diagnosed malignant disease (n = 10), and in existing patients treated with immunotherapy (n = 10). **Methods:** Parallel blood and stool samples were collected and used in the biomarker and microbiome analysis. The biomarkers in the two groups were analyzed by Principal Component Analysis, heat map with clustering, and differential expression based on *P* value, and Significance Analysis of Microarrays (SAM). The microbiome analysis was performed using long read 16S rRNA encoding gene sequencing with data visualization and analysis in *R*. Significant differences in alpha and beta diversity were evaluated between the groups. **Results:** Several biomarkers that were differentially expressed were identified. Significant taxa differences at the class (Clostridia), order (*Clostridiales*, *Lactobacillillales*), family (*Eubacteriaceae*, *Lactobacillaceae*), genus and species were identified. Furthermore, a limited analysis of samples from existing patients on immunotherapy who were responders (n = 4) *vs*. stable non-responders (n = 5) identified differentially expressed immune biomarkers and significant bacterial taxa differences. **Conclusion:** This study has established the feasibility for conducting a future larger study at the local community cancer center to evaluate whether immune and microbiome markers can predict effective *vs*. ineffective responses to immunotherapy and whether either or both molecular and microbial markers may have therapeutic potential.

Key words: immune biomarker, fecal microbiome, malignant solid tumors, feasibility study, immunotherapy, responder, progressor

***Corresponding Author:**
Gordan Srkalovic, Herbert-Herman Cancer Center, E W Sparrow Hospital, Lansing, MI 48912, United States.
E-mail: Gordan.Srkalovic@Sparrow.org. https://orcid.org/0000-0003-3519-04090
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INTRODUCTION

Biomarker discovery is an exciting area of scientific breakthroughs in various medical conditions including cancers, infectious diseases, autoimmune and allergic diseases.[1–4] Availability of the technology to screen for many immune biomarkers using relatively small quantities of human body fluids including the blood has resulted in enormous research efforts in this direction. Primary reasons of anticipated benefits from identification of biomarkers for cancers include their utility in the diagnosis, risk analysis, disease classification, prevention, treatment, decision making, and management of malignant diseases.[5–9] Furthermore, such knowledge is also expected to advance the mechanistic basis of diseases at the fundamental level.[1–4] Therefore, it is critical to advance immune biomarker discovery in various human diseases including malignant solid tumors, which is the focus of this study.

There is growing scientific evidence supporting the hypothesis that commensal microbial populations (*i.e.*, microbiome) present in humans have a significant impact on human health.[10–12] Numerous studies, both in humans and in animal models, demonstrate major impacts of specific populations of gut bacteria on the pathogenesis of cancer, obesity, allergies, asthma, and neurological disorders.[10–12] Consequently, large-scale efforts are underway to catalog the human microbiome both in Europe (European Metagenomics of the Human Intestinal Tract) as well as in the USA (the Human Microbiome Project).[10–12] Numerous studies demonstrate that the fecal microbiome profile is different not only in cancer *vs.* non-cancerous control subjects, but also in responder *vs.* non-responder cancer patients to various types of treatments in several types of malignant tumors.[13–25]

The first set of studies in this domain demonstrated that specific gut bacteria could positively influence the outcome of immunotherapy in mouse models of melanoma and colon cancer.[15–17] For example, supplementation of *Bifidobacteria* had the same degree of effect in controlling tumor burden as the efficacy of checkpoint inhibitor (anti-PD-L1) therapy in a mouse model of melanoma.[25] Furthermore, combination therapy with the bacteria plus the drug was able to eliminate tumor expansion.[25] Similar beneficial effects of another type of gut bacteria (*Bacteriodes*) have been reported in animal models of melanoma and colon cancer with anti-CTLA-4 therapy and cyclophosphamide therapy.[15,16] The underlying beneficial effect of these bacteria in cancers is mediated by potentiating natural anti-cancer immunity.[15–17,25] Two recent studies demonstrate that fecal transplantation from responders to non-responders in anti-PD-1 immunotherapy melanoma patients shows benefits and overcomes refractoriness to immunotherapy in a subset of treated patients.[26,27] However, this procedure is not readily available and could be costly (lowest cost $ 5250).[28]

Despite exciting recent advancements on the potential benefits of immune biomarkers and the gut microbiome patterns, very little is known about their relationships with the specific type of cancers or groups of cancers (*e.g.*, solid malignant tumors as a group). Furthermore, specific biomarkers and microbiome signatures to predict success or failure of different types of cancer immunotherapy protocols also remain to be clearly defined.

The long-term goal of this project is to elucidate the relationship among systemic immune biomarkers, fecal microbial signatures, and the outcome of cancer immunotherapy in patients treated at the Herbert-Herman Cancer Center in Lansing, Michigan. The present pilot study was conducted to establish the feasibility for conducting a future, larger, long-term study to advance our long-term goal. Such study will also inform the development of novel intervention methods to enhance therapeutic success in cancer patients. The overall hypothesis guiding this feasibility study was that different patterns of systemic immune markers and fecal microbiome profiles will be identified by conducting a pilot study using a small sample of subjects with and without malignant solid tumors as groups.

There were four specific objectives for this feasibility study: (i) to demonstrate successful patient recruitment for the study; (ii) to optimize methods to collect, transport and store fecal and blood samples from three groups of subjects: newly diagnosed with malignant solid tumors (N group), existing patients undergoing immunotherapy for solid tumors (E group), and healthy control subjects without malignant tumors (H group); (iii) to analyze immune markers in the plasma of these subjects; and (iv) to determine the fecal microbiome profile in these subjects. We report the identification of differential biomarkers and microbiome profiles in subjects without and with malignant tumors and establish the feasibility of conducting a future larger study.

MATERIALS AND METHODS

Subjects, approval of the protocol by the Institutional Review Boards, and recruitment

The project was approved by the Sparrow Health System Institutional Review Board (Lead IRB) and the Michigan State University (MSU) IRB. Newly diagnosed patients with malignant tumors (n = 10), existing patients undergoing immunotherapy (n = 10), and

Table 1: Demographics of subjects used in this study

Groups of subjects	Gender	Age (range, mean ± SD, median)	Types of solid malignancies
Newly diagnosed patients with malignancies (n = 10)	5F, 5M	53–91, 66 ± 11, 64	Adenocarcinoma of the lungs (3) Mixed histology lung cancer (1) Small cell lung cancer (1) Esophageal adenocarcinoma (1) Malignant melanoma (1) Head and neck squamous cell carcinoma (1) Ovarian adenocarcinoma (1) Bladder cancer (1)
Existing cancer patients on immunotherapy (n = 10)	5F, 5M	56–75, 66.4 ± 7.1, 65	Adenocarcinoma lungs (5) Squamous carcinoma lungs (1) Clear cell kidney cancers (2) Endometrial cancer (1) Merkel cell carcinoma (1)
Healthy subjects without malignancies (n = 10)	7F, 3M	38–83, 63 ± 14.7, 64	No malignancies

healthy subjects without malignant tumors (n = 10) were recruited for this study (Table 1). Existing patients with malignancies undergoing immunotherapy were treated with standard check point inhibitor protocols (NSCLC, melanoma, head and neck cancers, bladder and renal cancers and Hodgkin's Lymphoma). Good responders were considered all patient with partial response (PR) or better by Recist 1.1 criteria (Eisenhauer *et al.* 2009). Poor responders were patients with stable disease and responses that did not achieve PR.

The following inclusion criteria were used to enroll patients for this study: (1) Any new patients starting treatment for any malignancies with one of the following approved immune check point inhibitor therapies: Pembrolizumab, Nivolumab or Atezolizumab; (2) Patient could have a newly diagnosed disease or may have been previously treated with other lines of therapy including any chemotherapy or targeted treatments. There was no limit on previous lines of treatment; and (3) Any patient having equal or greater than 20% reduction in bidimensional measurements by RECIST 1.1 were called as responders for this study. Non responders were patients who had less than 20% reduction in measurements, stable or progressive disease.

Fecal samples were collected from new patients before starting the therapy, healthy subjects at the time of enrollment, and from existing patients during immunotherapy. Complete blood count (CBC), Comprehensive metabolic panel (CMP) and other standard of care clinical tests were done based on best clinical practice. Response to therapy was determined based on CT scan at 3 months and 6 months and then at every 3 months interval until progression or removal due to toxicities or patient choice. This was in accordance with Good Clinical Practice (GCP) and was standard of care for patients with malignancies treated with oncolytic treatment. Blood and fecal samples were collected in parallel from all subjects.

Consent: Informed written consent from each donor were obtained. Patients and healthy donors were provided the following information: (1) Collect stool in the plastic toilet hat (Fisher 02-544-208) and use the spatula in the specimen collection tube to put ~10 grams of the stool (fill ~ 3/4th full) into the plastic specimen bottle (Parapak, Meridian Bioscience 900312) and tightly close the lid. Write your name, date, and time of collection. Store at room temperature. Do not refrigerate the sample; store and transport the sample at room temperature only. Any deviations to this method must be reported when samples are delivered. Return the sample to the Cancer Center within 24 hours of sample collection; (2) Provide the diet information for the past 2 days prior to fecal donation. This information will be collected on a specific form (form enclosed) by the staff of Sparrow Cancer center's clinical trials office; (3) Provide the verification that antibiotics were not taken for at least 5 days prior to stool donation; and provide information on the antibiotics if they were used during the past month prior to fecal donation. This information will be collected on a specific form (form enclosed) by the staff of Sparrow Cancer center's clinical trials office; and (4) Provide information on the gender, ethnicity, age, and history of allergies (airways allergy, skin allergy and food allergies). For information on food allergies and food intolerances, donors will be asked to name the specific food or agent they are allergic or intolerant to. This information will be collected on a specific form (form enclosed) by the staff of Sparrow Cancer center's clinical trials office.

Blood collection, processing and biomarker analysis

Blood samples were collected, stored, and transported at 4°C to the immunology laboratory (Dr. Gangur) at MSU. The blood samples were processed within 24 hours of collection, plasma harvested and stored in

Table 2: The differentially expressed biomarker (s) identified in this study by SAM (delta = 1.2): comparison of subjects without malignant tumors *vs*. newly recruited cancer patients

Biomarkers	Healthy patients	Cancer patients	*D* value	Raw *P*	*Q* value
IP-10	39.36 ± 15.54	94.19 ± 52.82	-3.148935	0.0011	0.0134573
MCP-1	178.77 ± 34.67	276.64 ± 95.98	-3.042022	0.0011	0.0134573
HCC-4	425.51 ± 67.49	600.18 ± 125.25	-3.882261	0.0003	0.0055053
TIMP-1	1040.17 ± 113.27	1260.61 ± 106.65	-4.480754	0.0000	0.0000000
TIMP-2	2156.16 ± 295.97	2472.75 ± 185.36	-2.866803	0.0021	0.0220210
OPN	20585.44 ± 7558.66	32759.98 ± 106.65	-4.546563	0.0000	0.0000000
PF4	8315.03 ± 2199.71	12131.51 ± 2019.77	-4.041331	0.0001	0.0024468

Table 3: The differentially expressed biomarker(s) identified by SAM (with delta = 1.2): comparison of responders *vs*. stable non-responder subgroups within the existing patients treated with immunotherapy

Biomarkers	Responder (E1239)	Stable non-responder (E456810)	*D* value	Raw *P*	*Q* value
CXCL16	30636.78 ± 22183.49	3614.79 ± 6852.99	-3.349104	0.0030303	0.0542516
IFNg	822.51 ± 681.37	117.12 ± 85.28	-2.605509	0.0116162	0.0542516
ANG-2	6177.3 ± 2002.55	3591.18 ± 420.88	-2.533098	0.0129293	0.0542516
I-TAC	5809.71 ± 2619.55	1870.85 ± 1942.94	-2.502608	0.0140404	0.0542516
CTACK	4553.02 ± 2570.52	1222 ± 1145.46	-2.403843	0.0163636	0.0542516
MIP-3b	3157.85 ± 1872.02	915.2 ± 397.89	-2.348597	0.0179798	0.0542516
IL-17F	30636.78 ± 22183.49	3614.79 ± 6852.99	-2.347801	0.0180808	0.0542516
MCP2	375.99 ± 185.08	149.88 ± 94.94	-2.175568	0.0261616	0.0542516
MPIF-1	1559.2 ± 635.15	795.6 ± 329.94	-2.167492	0.0263636	0.0542516
TSLP	1653.41 ± 1018.09	361.56 ± 729.99	-2.128772	0.0287879	0.0542516
IL-31	3772.85 ± 3025.35	447.97 ± 876.59	-2.124841	0.0291919	0.0542516
MIF	7715.39 ± 5062.2	2258.39 ± 951.85	-2.124368	0.0292929	0.0542516
SDF-1a	202.74 ± 148.61	40.38 ± 38.61	-2.070678	0.0327273	0.0542516
BTC	717.9 ± 644.74	40.82 ± 91.27	-2.070200	0.0329293	0.0542516
IP-10	178.49 ± 123.42	46.08 ± 11.83	-2.067112	0.0335354	0.0542516
B7-2	3436.31 ± 2462.31	871.16 ± 386.8	-2.059756	0.0342424	0.0542516
LIF	822.51 ± 681.37	117.12 ± 85.28	-2.045022	0.0354545	0.0542516
IL-1a	201.66 ± 164.95	28.73 ± 25.46	-2.025479	0.0368687	0.0542516
TNFb	147.83 ± 133.46	9.62 ± 13.4	-1.999671	0.0387879	0.0542516
IL-6	109.76 ± 78.4	21.22 ± 6.84	-1.979169	0.0415152	0.0542516
IL-23	400.28 ± 364.99	31.14 ± 65.74	-1.974370	0.0425363	0.0542516

aliquots at −70°C. Plasma was used in the biomarker analysis using a protein microarray method (Ray Biotech Inc.). The core bioinformation group at the Ray Biotech analyzed all the immune biomarker data and determined the significance. The processes related to the analysis were as follows.

Data filtration: Biomarkers showing no variation across all the subjects *(i.e.*, zero-variance), were excluded from the analysis.

Data scaling for heatmap and principal coordinate analysis: The biomarkers values were centered and scaled by subtracting the mean of each biomarker from the data and then dividing it by the standard deviation, respectively. Centering and scaling results in a uniform mean and scale across all the biomarkers but leaves their distribution unchanged.

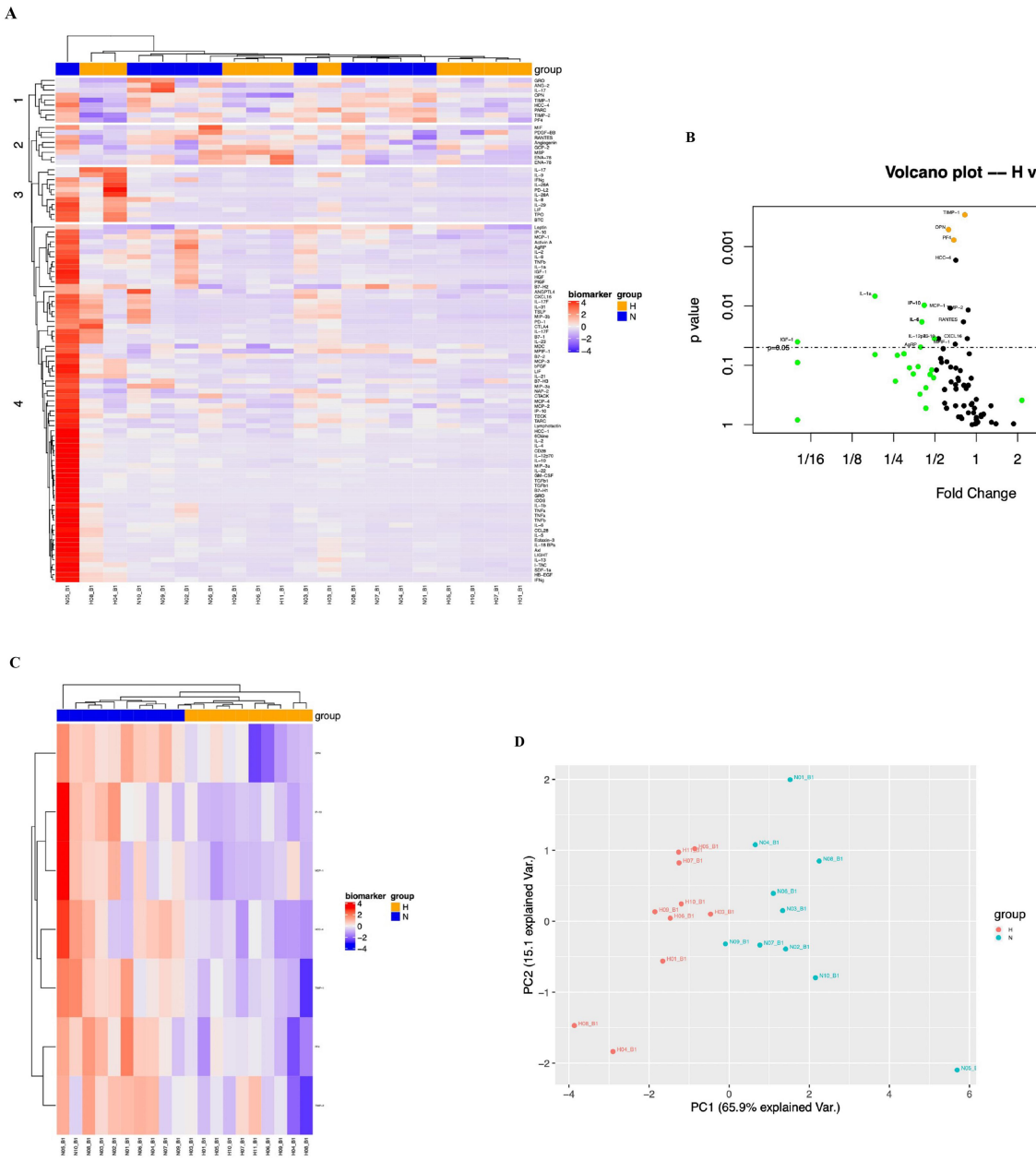

Figure 1. Analysis of immune biomarkers in subjects without malignant tumors (healthy subjects) *vs*. newly recruited subjects with malignant solid tumors (new patients). **A**. Heatmap of 100 biomarkers in healthy (H) subjects *vs*. new patients (N). **B**. Volcano plot comparing the fold change and *P* value of 100 biomarkers in healthy (H) subjects *vs*. new patients (N). Each point represents a biomarker. **C**. Heatmap of 7 differentially expressed biomarkers between healthy (H) subjects *vs*. new patients (N). **D**. PCA plots of 7 differentially expressed biomarkers between healthy (H) subjects *vs*. new patients (N). PCA: principal component analysis.

Principal component analysis: The scaled data were transformed with principal component analysis (PCA). Each principal component (PC) derived from PCA was a weighted summation of all the input measurements/biomarkers, in which the weights form a vector orthogonal to those of the other PCs. Thus, a sample with *P* biomarker values was transformed to a datapoint defined by PCs. The PCs were ordered by variation contained/explained by themselves, thus providing a good way for dimension reduction and pattern observation. Also, the biomarkers with large weights in a PC might share some common characteristics.

Heatmap with hierarchical clustering: The scaled and centered data were plotted as a heatmap in which the different colors represent biomarker expression levels. The biomarkers and samples were then subjected to hierarchical clustering based on Euclidean distance.

Comparison across groups: Each biomarker value was summarized by its mean and standard deviation, or median with minimum and maximum responses across

the groups. The fold change between groups was calculated as the ratio of the mean or median. If the biomarkers met or did not meet normality criteria across two groups, the significance of expression difference (FDR $P < 0.05$) was evaluated by the paired *t*-test or signed-rank test, respectively.

Significance analysis of microarrays: The significance analysis of microarrays (SAM) identifies differentially expressed biomarkers or genes.[29] Unlike the above-mentioned statistical tests that rely on *P* value from theoretical probability distribution, SAM compares a *t*-like statistic observed across groups with an estimate obtained from randomized permutations of the samples. The biomarkers with deviations larger than predefined Δ (default 1.2) were identified as differentially expressed.

Software: All analyses were conducted in the R programming language V3.6.3.[30] The SAM analysis was implemented with the R package siggenes 1.60.0.[31] The Pathway/GO over-representation and GSEA analyses were implemented with the R package clusterProfiler.[32]

Fecal sample-processing, and microbiome analysis

During collection and transportation to the clinic, all stool samples were at ambient temperature per the published method.[33–35] The Cancer Center personnel de-identified the samples so samples could be stored and analyzed in a blinded fashion. Samples were then transported on ice briefly (up to 2 hours) and stored at Michigan State University (Dr. Gangur's Immunology Laboratory). Stool samples were then stored in aliquots at −70°C. DNA extractions and fecal microbiome 16S rRNA gene sequencing was conducted by Loop Genomics Inc., which provided the taxonomic and count data to Dr. Sarah Comstock (Microbiome Laboratory, Michigan State University), who conducted further analysis using the published methods from her laboratory.[36–40] Sequence counts were rarified to 9999 reads per sample (performed 999 times, averaged, then rounded), and rarefaction curves confirmed adequate community coverage. Rarified count data was used in further analyses. Alpha (within participant) and beta (between participants by treatment group) diversity indices were used to characterize the gut microbial diversity. We quantified alpha diversity using three metrics, Chao1, Shannon, and Inverse Simpson. Each provides insight into the overall community diversity within an individual by accounting for richness (Chao1) or both richness and evenness (Shannon/Inverse Simpson). A higher score indicates a higher diversity. Beta diversity was visualized by plotting the Sorensen and Bray-Curtis dissimilarity scores on a principal coordinates plot. An individual is represented by a single point, and the closer two points are to each other, the more similar the respective gut microbial communities of those two individuals. Clustering of samples by group would suggest differences in community composition by group. To compare alpha diversity by group either ANOVA (normally distributed data) or Friedman Wilcoxon rank-sum (non-normally distributed data) tests were used. To compare beta diversity by group, PERMANOVA was used via the adonis function in the vegan package, and differences in group dispersion were determined using PERMDISP in the same R package.[41] To make taxa level comparisons by group, counts were compared across groups for each taxa using negative binomial regression. *P* values were false discovery rate corrected using the method of Benjamini-Hochberg.[42] All microbiota-related diversity and statistical analyses were conducted using *R*, a free statistical software program.

RESULTS

Identification of differentially expressed immune biomarkers between healthy vs. new cancer patients not yet treated

Subjects in this study were categorized into three groups depending on the specific types of malignancies they had (Table 1). To establish baseline differences in systemic immune biomarkers between the subjects with and without malignant tumors, comparisons were made. The biomarkers in the two groups were analyzed by Principal Component Analysis, heat map with clustering, and differential expression based on *P* value, and significance analysis of microarrays (SAM). The PCA showed that most variation of data was explained by PC1 (65.2%), and then PC1 (9.6%). The first two PCs presents the pattern of samples while keeping as much as information about variations across them.

Based on heatmap with hierarchical clustering, the 100 biomarkers were clustered into 4 groups (Figure 1A) by Euclidean distance after scaling and centering. The Figure 1B shows the volcano plot comparing the fold change and *P* value of 100 biomarkers in which each point represents a biomarker. As evident, the following 15 biomarkers were significantly different ($P < 0.05$) between these groups: IGF-1, MPIF-1, IL-12p70, IL-10, CXCL16, IL-6, RANTES, MCP-1, IP-10, IL-1a, TIMP-2, HCC-4, PF4, OPN, and TIMP-1. Blood levels of all these biomarkers were significantly higher in newly recruited cancer patients with malignant tumors.

The data was further tested by SAM (with delta 1.2) and identified the following 7 differentially expressed biomarkers: IP-10, MCP-1, HCC-4, PF4, OPN, TIMP-1, TIMP-2. The Figure 1C shows the heatmap analysis of 7 differentially expressed biomarkers between the two groups. The Figure 1D shows the PCA plots of 7 differ-

entially expressed biomarkers between the two groups. The Table 2 shows the comparison of 7 differentially expressed biomarkers between the two groups. As evident, these 7 immune biomarkers were at significantly higher levels in the blood of cancer patients at diagnosis compared to the healthy subjects without malignant tumors.

Comparison of immune biomarkers between healthy vs. existing cancer patients undergoing immunotherapy

The biomarkers in the two groups were analyzed by as above. One biomarker with zero variance (GRO) was excluded from PCA and heatmap analysis. The PCA showed that most variation of data was explained by PC1 (63.7%), and then PC2 (8.9%) (Figure S1A). Based on heatmap with hierarchical clustering, 99 biomarkers were clustered into 4 groups (Figure S1B) by Euclidean distance after scaling and centering. Figure S1C shows the volcano plot comparing the fold change and *P* value of 99 biomarkers in which each point represents a biomarker. As evident, the following 10 biomarkers were significantly different ($P < 0.05$) between these groups with higher levels noted in the cancer patients on immunotherapy: IGF-1, HCC-4, MIP-3b, IP-10, ICOS, CD28, PD-1, RANTES, OPN, NAP-2. However, based on testing by SAM (with delta 1.3) only 1 biomarker (NAP-2) was found differentially expressed between the 2 groups with higher levels noted in the cancer patients on immunotherapy (Figure S1D**).**

Identification of differentially expressed immune biomarkers in responder vs. stable subgroup of cancer patients on immunotherapy

Based on response to immunotherapy, the existing patients on immunotherapy consisted of 3 subgroups: responder ($n = 4$), non-responder stable ($n = 5$), non-responder progressor ($n = 1$). Therefore, to determine whether differences in systemic immune biomarkers could be identified between the responder *vs.* non-responder stable groups of cancer patients on immunotherapy, we conducted biomarker analysis as described above. One biomarker with zero variance (GRO) was excluded from PCA and heatmap analysis.

The PCA showed that most variation of data was explained by PC1 (70.6%), and then PC2 (10.4%) *etc.* (Figure 2A). Based on heatmap with hierarchical clustering, the 99 biomarkers were clustered into 4 groups (Figure 2B) by Euclidean distance after scaling and centering. Figure 2C shows the volcano plot comparing the fold change and *P* value of 9 biomarkers in which each point represents a biomarker. As evident, the following 2 biomarkers were significantly different ($P < 0.05$) between these groups: IL-8 and CXCL16. Higher levels were noted in the responder group compared to the non-responder stable group of patients.

The data was further tested by SAM (with delta = 1.7). This analysis identified 21 differentially expressed biomarkers shown in the heatmap analysis (Figure 2D): CXCL16, IFNg, ANG-2, I-TAC, CTACK, MIP-3b, IL-17F, MCP-2, MPIF-1, IL-6, TSLP, IL-31, MIF, SDF-1a, BTC, IP-10, B7-2, LIF, IL-1a, TNF-b, IL-23. Figure 2E shows the PCA plots of differentially expressed biomarkers between the two groups. Comparison of these 21 differentially expressed biomarkers between the two groups is shown in Table 3. All 21 immune markers were present at higher levels in responder group compared to the non-responder stable group of patients.

Fecal microbiome analysis between healthy vs. new cancer patients not yet treated

To establish baseline differences in the fecal microbiome profile between the subjects with (n) and without (h) malignant tumors, comparisons were made at the phyla, class, order, genus, and species levels. The two groups of subjects had significant differences in beta diversity at the phylum level (Sorensen metric, PERMANOVA, $P = 0.013$; PERMDISP, $P = 0.620$) (Figure 3A). The healthy group had a significantly higher alpha diversity at the class level (11.25 ± 2.62) compared to the cancer patients (8.8 ± 2.10) (Chao 1 metric, $P < 0.05$)**.**

The following taxa were significantly different at the class level: *Clostridia* (higher in healthy subjects, $P < 0.05$), and possibly *Bacilli* (higher in cancer patients, $P = 0.05$) (Table 4). At the order level, *Clostridiales* were higher in healthy subjects ($P < 0.05$), and possibly *Lactobacillales* were higher in cancer patients ($P = 0.052$) (Table 4). At the family level, *Eubacteriaceae* were higher in healthy subjects ($P < 0.01$) and *Lactobacillaceae* were higher in cancer patients ($P < 0.01$) (Table 4).

At the genera level, the two groups of patients showed significant differences in the following 7 genera ($P < 0.05$): whereas *Clostridium, Dorea, Intestinibacter, and Lactobacillus* were higher in cancer subjects*, Catenibacterium*, *Erysipelotrichaceae,* and *Subdoligranulum* were higher in healthy subjects (Table 4). At the species level, whereas *Clostridium perfringens, Intestinibacteria* sp., and *Lactobacillus salivarius* were higher in cancer subjects*, Catenibacterium* sp.*, Erysipelotrichaceae* sp., and *Subdoligranulum* sp. were higher in healthy subjects (Table 4).

Fecal microbiome analysis between responder vs. stable cancer patients treated with immunotherapy

To establish the possible differences in the fecal microbiome profile between the cancer patients on immunotherapy that are responders ($n = 4$) *vs.* non-

Table 4: Taxonomic abundance in fecal samples collected from subjects without malignant tumors (healthy) *vs.* newly recruited patients with malignant solid tumors (cancer)

	Healthy patients	Cancer patients	*P* value
Class			
Bacilli	2.5 ± 3.4	12.7 ± 16.4	0.050
Bacteroidia	1.9 ± 3.2	1.2 ± 1.5	0.812
Clostridia	66 ± 30.5	57.1 ± 32.6	< 0.0001
Erysipelotrichia	7 ± 9.7	6.1 ± 4.3	0.925
Gammaproteobacteria	18.3 ± 35.8	19.9 ± 38.2	0.925
Verrucombicrobiae	3.3 ± 4.9	1.6 ± 3.4	0.812
Order			
Bacteroidales	1.9 ± 3.2	1.2 ± 1.6	0.812
Clostridiales	66 ± 30.5	57.1 ± 32.6	< 0.0001
Enterobacteriales	18.2 ± 35.8	19.9 ± 38.1	0.926
Erysipelotrichales	7 ± 9.7	6.1 ± 4.3	0.926
Lactobacillales	2.5 ± 3.3	12.7 ± 16.4	0.052
Verrucomicrobiales	3.3 ± 4.9	1.6 ± 3.4	0.812
Family			
Akkermansiaceae	3.3 ± 4.9	1.6 ± 3.4	0.677
Clostridiaceae 1	0.5 ± 1	6.9 ± 14	0.188
Enterobacteriaceae	18.2 ± 35.8	19.9 ± 38.1	0.926
Erysipelotrichaceae	7 ± 9.7	6.1 ± 4.3	0.863
Eubacteriaceae	0 ± 0.1	2.4 ± 5	0.007
Lachnospiraceae	54.2 ± 27.3	35.9 ± 32.5	0.562
Lactobacillaceace	0 ± 0.1	5.6 ± 14.7	0.010
Peptostreptococcaceae	1.2 ± 2.4	6.6 ± 11.7	0.214
Ruminococcaceae	9.2 ± 7.3	3.4 ± 3.6	0.214
Streptococcaceae	2.4 ± 3.2	6.5 ± 10	0.265
Genus			
Agathobacter	1+2.9	1.7 ± 3.5	0.724
Akkermansia	3.3 ± 4.9	1.6 ± 3.4	0.715
Anaerostipes	1.7 ± 1.7	2.5 ± 4.5	0.724
Blautia	30 ± 16.6	14.1 ± 13.2	0.220
Cantenibacterium	2.6 ± 8.2	0.2 ± 0.6	< 0.001
Clostridium	0.5 ± 1	7.9 ± 15	0.020
Dorea	3 ± 3.1	3.7 ± 5.5	< 0.0001
Erysipelotrichaceae	2.6 ± 3.8	1.8 ± 4.2	< 0.0001
Escherichia Shigella	18.2 ± 35.8	18.5 ± 38.7	0.986
Eubacterium	9.7 ± 6.7	7.7 ± 11.3	0.765
Intestinibacter	0 ± 0	2.8 ± 6	0.004
Lachnoclostridium	1.8 ± 2.1	2.8 ± 4.9	0.715
Lactobacillus	0 ± 0.1	5.6 ± 14.7	0.005
Romboutsia	1.2 ± 2.4	3.4 ± 6	0.497
Ruminococcaceae	2.9 ± 2.7	1.1 ± 0.8	0.175
Ruminococcus	4.2 ± 4	2.4 ± 3.6	0.561
Streptococcus	2.3 ± 3.2	6.5 ± 10	0.276
Subdoligranulum	3.7 ± 3.5	1.8 ± 2.7	< 0.0001
Species			
Agathobacter sp.	1 ± 2.9	1.7 ± 3.5	0.794
Akkermansia sp.	3.3 ± 4.9	1.6 ± 3.4	0.732

(To be Continued)

(Continued)

Anaerostipes sp.	1.1 ± 1	1.5 ± 2.4	0.799
Blautia sp.	28.2 ± 16.2	13.3 ± 12.6	0.197
Catenibacterium sp.	2.6 ± 8.2	0.2 ± 0.6	< 0.001
Clostridium perfringens	0 ± 0	2.1 ± 6.6	< 0.001
Clostridium sp.	0.5 ± 1	2.8 ± 5	0.147
Dorea Longicatena	1.1 ± 1.4	1.1 ± 1.9	0.159
Dorea sp.	1.8 ± 2.2	2.4 ± 3.6	0.814
Erysipelotrichaceae sp.	2.6 ± 3.8	1.8 ± 4.2	1.925
Escherichia coli	18.2 ± 35.7	18.5 ± 38.6	0.986
Eubacterium sp.	9.7 ± 6.7	7.7 ± 11.3	0.799
Intestinibacter sp.	0 ± 0	2 ± 4.7	0.002
Lachnoclostridium sp.	1.8 ± 2.1	2.8 ± 4.8	0.732
Lactobacillus salivarius	0 ± 0	3.8 ± 12.1	< 0.001
Romboutsia sp.	1.1 ± 2.3	3.1 ± 5.5	0.503
Ruminococcaceae sp.	2.9 ± 2.7	1.1 ± 0.8	0.159
Ruminococcus sp.	3.6 ± 3.4	1.2 ± 1.1	0.147
Streptococcus salivarius	0.7 ± 1.4	1.5 ± 2.9	0.646
Streptococcus sp.	0.5 ± 0.8	1.9 ± 2.7	0.147
Subdoligranulum sp.	3.7 ± 3.5	1.8 ± 2.7	< 0.0001

Taxa below average relative abundance of 1% are excluded.

responder (stable) (n = 5) microbiome analysis was conducted. The two groups of subjects had no significant differences at the phylum level. The alpha diversity at the class level (Shannon and Inverse Simpson) were significantly different between the two groups ($P < 0.05$). The Shannon value for the stable group was 1.08 ± 0.366, while it was 0.441 ± 0.136 for the responder group. Additionally, the Inverse Simpson was 2.309 ± 0.809 for the stable group, but it was 1.235 ± 0.125 for the responders. *Clostridia* was the only taxa that showed significant differences between the groups, with higher levels noted in the responder group ($P < 0.05$) (Table 5). At the order level alpha and beta diversity were significantly different between the responder group compared to the stable group. The order *Clostridiales* was more dominant in the fecal bacterial communities of the responder group (Figure 3B). The Shannon value for the stable group was 1.09 ± 0.369, while it was 0.443 ± 0.136 for the responder group. Meanwhile, the Inverse Simpson was 2.311 ± 0.810 for the stable group, but 1.235 ± 0.124 for the responder group. In the responder group significantly higher levels of *Clostridales* were observed ($P < 0.05$) (Table 5). At the family level, significantly lower levels of *Streptococcoceae* were observed in the responder group (Table 5).

At the genus level, the two groups of patients showed significant differences in the following 8 genera ($P < 0.05$): whereas *Blautia, Christensenellaceae, Clostridium, and Dorea* were higher in the responder group, *Erysipelotrichaceae, Streptococcus, Anaerostipes,* and *Subdoligranulum* were higher in the stable group (Table 5). At the species level, the two groups of patients showed significant differences in the following 8 species ($P < 0.05$): whereas *Blautia sp., Christensenellaceae sp.,* and *Dorea longicatena* were higher in the responder group, *Erysipelotrichaceae sp., Ruminococcus gnavus, Streptococcus sp., Anaerostipes sp.,* and *Subdoligranulum sp.* were higher in the stable group (Table 5).

DISCUSSION

The primary goal of this study was to establish the feasibility of conducting a pilot study to investigate the immune biomarkers and fecal microbiome signature in groups of healthy subjects *vs.* subjects with solid malignancies, and in a small group of patients with solid malignancies who responded differently to immunotherapy at the local community cancer center. We tested the hypothesis that differential blood immune biomarkers and fecal microbiome profiles will be identified in this study. Our results collectively support this hypothesis.

For immune biomarker analysis, we selected 100 makers based on their roles in cancer biology, angiogenesis, immune responses, and inflammation. We analyzed their levels by 2 independent methods so that consistent and substantial differences between the compared groups could be identified. Based on fold change analysis, we identified significantly higher levels of 15 immune

Table 5: Taxonomic abundance in fecal samples collected from existing cancer patients on immunotherapy who were stable non-responders *vs*. responders

Class	Stable non-responder patients	Responder patients	*P* value
Bacilli	7.7 ± 10.3	2.4 ± 1.9	0.122
Bacteroidia	7 ± 6.1	2.1 ± 2.7	0.154
Clostridia	64.3 + 15.8	89.9 ± 4.8	0.016
Erysipelotrichia	10.4 ± 6.7	4.1 ± 4.3	0.122
Gammaproteobacteria	2.2 ± 4.1	0.5 ± 0.7	0.141
Verrucombicrobiae	5.2 ± 9.3	0.2 ± 0.3	0.122
Order			
Bacteroidales	7 ± 6.1	2.1 ± 2.7	0.160
Clostridiales	64.3 ± 15.8	89.9 ± 4.8	0.016
Enterobacteriales	2.1 ± 4.1	0.5 ± 0.7	0.160
Erysipelotrichales	10.4 ± 6.7	4.1 ± 4.3	0.122
Lactobacillales	7.7 ± 10.2	2.4 ± 1.9	0.122
Verrucomicrobiales	5.2 ± 9.3	0.2 ± 0.3	0.122
Family			
Akkermansiaceae	5.2 ± 9.3	0.2 ± 0.3	0.160
Bacteroidaceae	3.9 ± 3.1	1 ± 1.2	0.160
Christensenellaceae	1.7 ± 1.9	2.2 ± 2.8	0.917
Clostridiaceae 1	0.2 ± 0.4	6.1 ± 12.2	0.160
Enterobacteriaceae	2.1 ± 4.1	0.5 ± 0.7	0.253
Erysipelotrichaceae	10.3 ± 6.7	4.1 ± 4.3	0.160
Lachnospiraceae	46 ± 16.9	63 ± 26.3	0.253
Peptostreptococcaceae	5.5 ± 11.8	4.8 ± 7.9	0.919
Rikenellaceae	1.5 ± 1.7	0.6 ± 1	0.581
Ruminococcaceae	10 ± 5.6	11.3 ± 13.8	0.917
Streptococcaceae	7.5 ± 10.2	0.9 ± 0.4	0.0197
Genus			
Akkermansia	1.5 ± 1.7	0.2 ± 0.3	0.124
Alistipes	1.5 ± 1.7	0.6 ± 1	0.575
Anaerostipes	5.1 ± 4.3	0.7 ± 0.5	0.008
Bacteroides	3.9 ± 3.1	1 ± 1.2	0.124
Blautia	21.3 ± 5.6	35.4 ± 13.8	0.020
Christensenellaceae	1.7 ± 1.9	2.2 ± 2.8	3.226e-05
Clostridium	0.4 ± 0.4	6.2 ± 12.1	0.020
Dorea	1.1 ± 2	3.3 ± 4.6	8.967e-87
Erysipelatoclostridium	5.7 ± 6.2	2.7 ± 5.1	0.575
Erysipelotrichaceae	1.8 ± 2.1	0.6 ± 0.7	1.537e-49
Eubacterium	2.3 ± 2.2	6.1 ± 2.6	0.133
Lachnoclostridium	5.8 ± 9.9	9.9 ± 12.3	0.632
Romboutsia	5.2 ± 11.3	3.5 ± 5.4	0.785
Ruminococcaceae	3.3 ± 2.4	5.3 ± 7.2	0.575
Ruminococcus	6.7 ± 4.5	7.6 ± 5.7	0.785
Streptococcus	7.5 ± 10.2	0.9 ± 0.4	0.005
Subdoligranulum	3.2 ± 3	2 ± 3.5	9.586
Species			
Akkermansia sp.	5.2 ± 9.3	0.2 ± 0.3	0.114
Anaerostipes sp.	4.1 ± 3.9	0.5 ± 0.4	0.005
Bacteroides sp.	2.3 ± 2.3	0.5 ± 0.3	0.114

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Blautia sp.	20.7 ± 5	34.4 ± 13.5	0.014
Christensenellaceae sp.	1.7 ± 1.9	2.2 ± 2.8	1.970e-05
Dorea longicatena	0.6 ± 1.3	1.6 ± 2.8	7.634e-42
Erysipelatoclostridium sp.	5.7 ± 6.2	2.7 ± 5.1	0.6197
Erysipelotrichaceae sp.	1.8 ± 2.1	0.6 ± 0.7	6.437e-49
Eubacterium sp.	2.3 ± 2.2	6.1 ± 2.6	0.133
Lachnoclostridium sp.	5.8 ± 9.9	9.9 ± 12.3	0.632
Romboutsia sp.	4.8 ± 10.5	3.4 ± 5.3	0.812
Ruminococcaceae sp.	3.3 ± 2.4	5.3 ± 7.1	0.6197
Ruminococcus gnavus	1.8 ± 2.8	0 ± 0.1	0.013
Ruminococcus sp.	4.3 ± 3.8	5.7 ± 3.3	0.632
Ruminococcus torques	0.6 ± 1.4	1.6 ± 2.4	0.654
Streptococcus sp.	5.9 ± 10.6	0.3 ± 0.2	0.003
Subdoligranulum sp.	3.2 ± 3	2 ± 3.6	1.0697e-23

$P < 0.05$, and taxa below average relative abundance of 1% are excluded.

biomarkers in the blood of newly diagnosed cancer patients with malignant solid tumors compared to healthy subjects without solid malignant tumors: IGF-1, MPIF-1, IL-12p70, IL-10, CXCL16, IL-6, RANTES, MCP-1, IP-10, IL-1a, TIMP-2, HCC-4, PF4, OPN, and TIMP-1. Out of these, based on SAM, only 7 immune markers met the criteria of differential expression between healthy *vs.* cancer patients with substantially higher levels in the newly diagnosed patients with solid malignancies: IP-10 (CXCL10), MCP-1 (CCL2), HCC-4 (CCL16), PF4 (CXCL4), OPN, TIMP-1, TIMP-2. Wei *et al.* (2017) and Koopman *et al.* (2004) reported that higher serum OPN level was a promising diagnostic marker of pancreatic adenocarcinoma.[43,44]

There are several previous studies that report the utility of some of the above identified immune markers in the diagnosis and prognosis of specific cancers. Hefler *et al.* (1999) reported that higher blood MCP-1 distinguished benign from malignant ovarian cancer.[45] Narita *et al.* (2016) reported higher IP-10 and MCP-1 levels were associated with breast cancer metastasis.[46] Sorensen *et al.* (2007) reported that high blood levels of TIMP-1 in colorectal cancer reduced survival chances and predicted poor response to chemotherapy.[47] Miyake *et al.* (2014) reported that higher TIMP-2 levels were related to good response to sunitinib treatment in renal cell carcinoma patients. It is important to note that the cancer patients included a variety of solid malignant tumors.[48] Therefore, these immune markers are associated broadly with the diagnosis of new malignant tumors, rather than a specific type of cancer. Thus, they may represent the baseline differential immune markers to consider evaluating the impact of and response to immune therapy in patients with malignant solid tumors in future studies.

We found that 10 immune biomarkers were significantly higher in the cancer patients undergoing immunotherapy compared to the newly diagnosed patients: IGF-1, HCC-4, MIP-3b, IP-10, ICOS, CD28, PD-1, RANTES, OPN, NAP-2. Notably, IGF-1, HCC-4, IP-10, RANTES, OPN may be persistent biomarkers of solid malignant tumors as they are noted at high levels in newly diagnosed patients not yet treated, as well in patients who are already on immunotherapy. The other 5 biomarkers (MIP-3b, ICOS, CD28, PD-1 and NAP-2) were present at substantially higher levels among the treated patents only but not in untreated newly diagnosed patients with solid malignant tumors. This suggest that they may represent biomarkers that appear upon immunotherapy.

Since the existing patients on immunotherapy consisted of 2 subgroups based on their response to treatment, we conducted analysis between the responder group of patients *vs.* the stable group of patients. One patient was a non-responder progressor, and therefore was not included in this analysis. With this limited analysis with a very small sample size (n = 4–5), we found candidate immune markers associated with response to immunotherapy in cancer patients in this pilot study. Based on fold change analysis, higher levels of CXCL16 and IL-8 were noted in responders to immunotherapy. Our finding on IL-8 contrasts with a previous study that report that higher levels of IL-8, are associated with non-response in melanoma patients on immunotherapy.[49] In our cohort, there were no melanoma patients. Further studies are needed to evaluate whether the relationship between IL-8 levels and response to immunotherapy is different in different types of cancers.

The chemokine CXCL16 that uses the receptor CXCR6 was consistently identified to be significantly higher levels in the responder in both fold change analysis and the SAM. There are no reports of CXCL16 associated with response to immunotherapy in cancer. Previous studies show that higher levels CXCL16 is expressed by the breast cancer tissue upon radiation therapy, and it plays a critical role in recruiting NK cells to the cancer site.[50] In a mouse model of colon cancer, CXCR6 (the receptor for CXCL16) was required for intra-tumoral CD8 T cell killer activity.[51] Thus, CXCL16 may be an important biomarker of protection from cancer.

Our data analysis based on SAM show that there were 21 other markers besides CXCL16 that were present at significantly higher levels in responder group. Theis included cytokines (IFN-g, IL-17F, IL-6, TSLP, IL-31, MIF, LIF, IL-1a, IL-23 and TNF-b), chemokines [IP-10 (CXCL10), I-TAC (CXCL11), CTACK (CCL27), MIP-3b (CCL19), MCP-2 (CCL8), MPIF-1 (CCL23), SDF-1a (CXCL12)], an antigen presenting cell surface molecule (B7-2), and growth factors (BTC, ANG-2). Given the sample size used in this study, it would be necessary to follow-up on these markers and establish or rule out their role as biomarker of successful response to immunotherapy in future studies.

In this pilot study we found interesting differences in the fecal microbiome composition in newly diagnosed patients with malignant solid tumors compared to healthy subjects without solid tumors. We found that at both the genus as well as the species level *Clostridium perfringens, Intestinibacteria sp.*, and *Lactobacillus salivarius* were higher in cancer subjects. It was surprising that *Lactobacillus*, a genus that is associated with probiotic activities is higher in cancer subjects. Probiotic bacteria are generally associated with health and protection from diseases including colon cancer.[13] Nevertheless, our finding is consistent with recent studies that show that *Lactobacillus* may promote certain type of cancers. For example, a recent report shows that *Lactobacillus* can alter macrophage function in pancreas and promote cancer growth.[52] Another study shows that gastric cancer is associated with overgrowth of *Lactobacillus* bacteria in the stomach.[19]

Our very limited analysis of microbiome in responder *vs.* stable group of patients showed that *Blautia sp., Christensenellaceae sp.,* and *Dorea longicatena* were higher in the responder group. *Erysipelotrichaceae sp., Ruminococcus gnavus, Streptococcus sp., Anaerostipes sp.,* and *Subdoligranulum sp.* were higher in the stable group. There are no reports of microbiome profile in responder *vs.* non-responders to immunotherapy across the solid cancers as a group.

Previous reports all studied microbiome changes in specific types of solid malignancies as follows: Peng *et al.* (2020) reported that *Eubacterium, Lactobacillus*, and *Streptococcus*, were positively associated with anti-PD-1/PD-L1 response across different GI cancer types.[18] Other studies showed that responders to anti-PD1 therapy in cancers show abundance of bacteria as follows: *Akkermansia* In NSCLA and RCC, hepatocellular carcinoma;[20,22] *Clostridiales* in melanoma,[23] *Ruminococcaceae* in melanoma,[23] and hepatocellular carcinoma;[20] *Faecalibacterium* in melanoma;[23] *Bifidobacterium* in melanoma;[24] *Collinsella* in melanoma;[24] *Enterococcus* in melanoma;[24] *Alistipes putredinis* in NSCLC;[21] *Bifidobacterium longum* in NSCLC and *Prevotella copri* in NSCLC.[21] It is important to note that these are specific types of solid malignancies, and our feasibility study patients included several types of solid malignancies. Therefore, comparison with these results will not be meaningful at this time. We noted increased *Ruminoccous gnavus* in stable patients but not in the responder group and our study did not separately study cancer types. We did not note any differences in *Akkermansia* between responder *vs.* stable groups of patients in our study.

It is very important to note that the comprehensive list of gut bacteria associated with beneficial response to immunotherapy in cancers is only beginning to be understood. Furthermore, the association of bacteria can differ in different cohorts, and even within a given type of cancer. The approach of looking at gut bacteria broadly in responders *vs.* stable patients with solid malignant tumors on immunotherapy has not been reported before.

The primary goal of our pilot study was to demonstrate the feasibility of conducing this type of cancer research in a community cancer center. Given the budget and resources available, we recruited patients with several types of solid malignancies for this study. Our results demonstrate that despite the broad approach we used for this study, surprisingly significant differences were noted in both biomarkers and microbial profile. Kim *et al.* (2021) reported validation of a combined biomarker for prediction of response to checkpoint inhibitors in patients with advanced cancer of several types (*i.e.*, pan-cancer patients).[5] This raises the interesting possibility that differential biomarkers might become available that identify solid malignant tumors as a group (*i.e.*, pan-cancer biomarker concept). However, future large-scale studies are needed to test this hypothesis. In such future studies, it will also be important to establish correlations among specific fecal microbiome, specific immune marker (*e.g.*, cytokine, chemokine, growth factors) and the immunotherapy outcome because such correlations will provide insight into the mechanisms underlying the impact of immunotherapy.

The significance of biomarker discovery including microbiome markers in precision and personalized

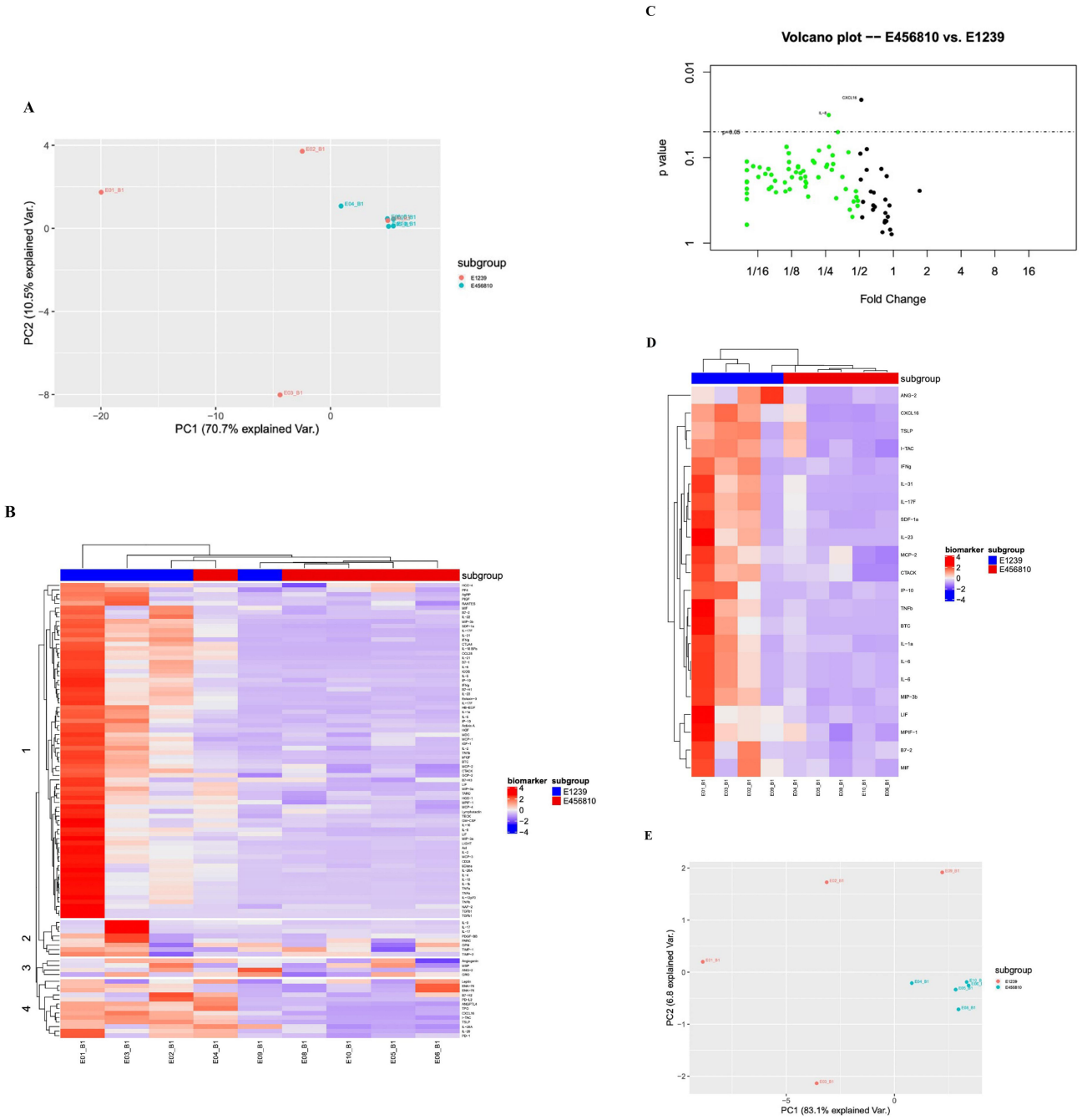

Figure 2. Analysis of immune biomarkers in subjects with malignant solid tumors receiving immunotherapy (Existing patients on immunotherapy): Comparison of responder (E1239) *vs*. stable (456810) subgroups. **A.** Plot of PC1 and PC2 values in responders (E1239) and stable (E456810) subgroups of existing patients treated with immunotherapy (E). **B.** Heatmap of 99 biomarkers in responders (E1239) and stable (E456810) subgroups of existing patients treated with immunotherapy (E). **C.** Volcano plot comparing the fold change and *P* value of 99 biomarkers in responders (E1239) and stable (E456810) subgroups of existing patients treated with immunotherapy (E). Each point represents a marker. **D.** Heatmap of 21 differentially-expressed biomarkers between responders (E1239) and stable (E456810) subgroups of existing patients treated with immunotherapy (E). **E.** PCA plots of 21-differentially expressed biomarkers between responders (E1239) and stable (E456810) subgroups of existing patients treated with immunotherapy (E). PCA: principal component analysis.

medicine in general and in oncology, cannot be understated. There are extensive efforts underway to identify specific biomarkers using omics technology and big data science approaches.[2–4] There are several reasons why this is an exciting future for the medical field including the following: (i) identification of superior biomarkers of effective *vs*. in effective and even harmful responses to treatment regimens will advance the precision medicine objective in immunotherapy and other types of treatments; (ii) combination of traditional markers and novel blood and fecal markers may assist in improving classification, and grading of cancers; for

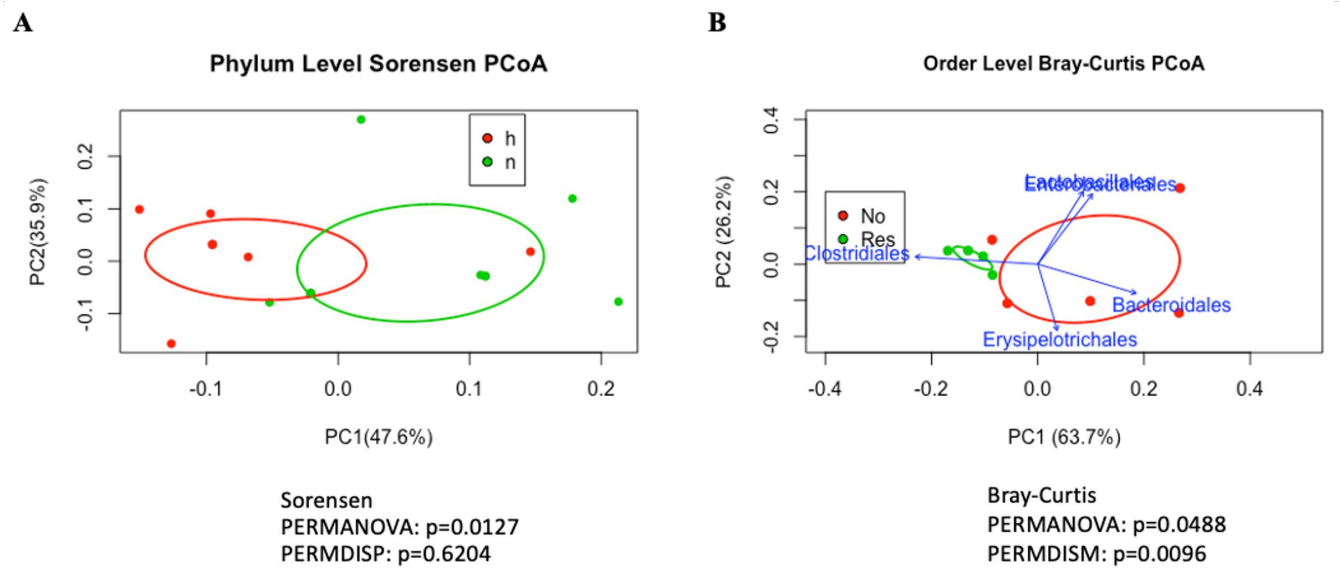

Figure 3. Analysis of beta diversity of fecal microbiome. **A.** Beta diversity based on the Sorensen dissimilarity metric. The fecal bacterial communities of patients with (cancer) (new patients, n) differed from those of subjects without (healthy) malignant colorectal tumors (healthy, h). **B.** Beta diversity based on the Bray-Curtis dissimilarity metric. The fecal bacterial communities of cancer patients on immunotherapy who were responders to immunotherapy (Res) included more *Clostridiales* compared to those of stable non-responder patients (No). PC: principal component.

example, Li *et al.* (2017) reported that conventional histopathological classification of breast cancer falls short of providing adequate prognostic and predictive power, and biomarkers may change this situation favorably;[9] and (iii) biomarkers may help in decision making for treatment duration for costly and long-lasting types of regimens.[2–4,6,7]

In conclusion, here we report the successful completion of a pilot study demonstrating the feasibility of conducting a research study in the local community cancer center in Lansing in collaboration with the Michigan State University. Therefore, this pilot study has established the feasibility for conducting a future larger study to evaluate whether immune and microbiome markers can predict effective vs. ineffective responses to immunotherapy and whether either immune biomarkers or microbe markers or both may have therapeutic potential.

SUPPLEMENTARY INFORMATION

Figure S1. Analysis of immune biomarkers in subjects without malignant tumors (healthy) *vs.* existing cancer patients on immunotherapy.

Supplementary information of this article can be found online at https://www.hksmp.com/journals/gfm/article/view/529/787

DECLARATIONS

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Author contributions
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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data sharing statement

No additional data are available.

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