TITLE PAGE

"Case-control study highlights a different gut microbiome in cirrhotic patients with and without hepatocellular carcinoma".

Short Title: "*Microbiome and hepatocellular carcinoma".*

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Abbreviations:

AFP: Alpha-fetoprotein; BMI: Body Mass Index; CC: cryptogenic cirrhosis; CI: Confidence Interval; CT: Computerized Tomography; HCC: Hepatocellular

carcinoma; HR: Hazard ratio; IQR: interquartile range; LT: Liver Transplantation; MC: Milan criteria; MELD: Model for End-stage Liver Disease; MRI: Magnetic Resonance Imaging; NAFL: Non-alcoholic fatty liver; NAFLD: non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; NLR: NOD-like receptors; OTUs: Operational Taxonomic Units; PEI: Percutaneous ethanol injection; RFA: radiofrequency ablation; TACE: trans-arterial chemoembolization; TLR: Toll-like receptors.

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ABSTRACT PAGE

Background & Aims: No specific microbiome in patients with hepatocellular carcinoma (HCC) has been reported to date. Our aim was to compare gut microbiome found in cirrhotic patients with and without HCC.

Methods: From 407 patients with Child Pugh A/B cirrhosis prospectively followed, 25 with HCC (cases) were matched with 25 without HCC (wo-HCC) in a 1:1 ratio according to age, gender, etiology, Child Pugh and severity of portal hypertension. In addition 25 healthy subjects were also compared. Faecal stool samples were collected noninvasively, aliquoted for DNA extraction and sequenced for the V3-V4 region of the microbial 16S rRNA (Illumina MiSeq Platform).

Results: Cases and control's baseline characteristics were: age 64 ± 8 years, 88% males, body mass index (BMI) 28 ± 4 kg/m2, hepatitis C virus infection 26%, Child Pugh A/B 74% and 26%, respectively. Barcelona Clinic Liver Cancer stages in HCC patients were 0 (n=2), A (n=12), B (n=5) and C (n=6). A significant different microbiome was observed in HCC cases vs controls. Patients with HCC had lower quantities of genus Prevotella, Fusobacterium and family Leuconostocaccaceae among others and an elevated proportion of genus Bifidobacterium, Eggerthella, Haemophilus, family Erysipelotrichaceae and phylum Tenericutes when compared to controls without HCC. This pattern has been associated with an inflammatory milieu with a putative increased activation of NOD-like receptor signalling pathways.

Conclusions: A different pattern of microbiome in cirrhotic patients with HCC was observed. This pattern previously linked to inflammation, should be further explored as a novel risk factor for HCC development.

Key words: Liver cancer – Microbiome – Inflammation

Introduction.

Increasing interest has been focused during the last years regarding microbiome and human diseases including cirrhosis, alcoholic liver disease, fatty liver and fibrosis progression. Changes in gut microbiome have been observed with progression of liver disease [1-4] including a reduced abundance of taxa considered benign, such as Lachnospiraceae, Ruminococcaceae and Clostridialies and a higher abundance of Enterobacteriaceae and Bacteriodaceae [2]. This gut microbiome profile remains stable unless a decompensation of cirrhosis appears with a relative increase in Enterobacteriaceae and Bacteriodaceae [2,5].

A renewed novel research has focused on microbiome and cancer development. However, scarce data has been written regarding microbiome and hepatocellular carcinoma (HCC). In patients with cirrhosis, there is an annual risk rate between 1 to 6% of developing HCC [6-8]. Previous studies showed that the severity of liver disease and chronic inflamation [9] are predictors for development of this tumor [10,11]. These changes have been linked to an inflammatory pro-oncogenic microenvironment from the intestine to the liver in murine models [12].

Inflamatory signals derived from a change in intestinal microbioma has been proposed as a novel carcinogenic mechanism [12-14]. This gut-liver axis may also become a potentially preventive therapeutic target [15,16]. The aim of this study was to evaluate the gut microbiome found in patients with HCC and to understand whether or not there is a specific gut microbiome profile among cirrhotic patients with HCC when compared to those without HCC.

Patients and Methods

Study design, setting and participating centers

This observational case-control study was nested on a prospective longitudinal cohort of patients with cirrhosis who were followed-up in our liver unit at Austral University Hospital, School of Medicine; in colaboration with HERITAS (Rosario), CONICET and the National Academy of Medicine. This study was carried out between December 2015 and October 2016 in accordance with international recommendations for observational studies [17].

Eligibility criteria

A consecutive non-probability sampling of adult subjects (>17 years old) with clinical or histological diagnosis of cirrhosis, functional status Child Pugh class A or B was included. Clinical or histological diagnosis of cirrhosis was done according to international consensus guidelines [18]. Exclusion criteria consisted of any of the following: a) Previous liver transplant, b) patient under any immunosuppressive treatment, c) prior or current treatment with any pre- or probiotic, d) active alcoholism (cessation of alcohol intake at least 3 months prior to study entry), e) past or present history of neoplasms, f) any major surgery or severe traumatic injury within 28 days prior to inclusion, g) active infection grade >2, according to the NCI CTCAE criteria, version 4.0 [19], h) infection with human immunodeficient virus (HIV), i) any antibiotic treatment should have been completed one month prior to the inclusion, excluding primary or secondary prophylaxis for bacterial infections in cirrhosis, j) diarrhea secondary to any commensal, including Clostridium difficile diarrhea within 6 months prior to the inclusion of the subject in the study, k) any malabsorption disorder, celiac disease or inflammatory bowel disease including ulcerative colitis and Crohn's disease.

Selection of cases and controls

Cases were defined as those patients meeting the above eligibility criteria and with imaging or histological diagnosis of HCC. Imaging HCC diagnosis was performed with a tri-phase dynamic study, either Computerized Axial Tomography (CT) or Magnetic Resonance Imaging (MRI) as recommended by international guidelines [20,21]. All cases were allowed to have a history of HCC treatment, excluding liver transplantation. They should have at least one active HCC nodule [22] at time of fecal stool sample collection. Images were centrally reevaluated by a single observer, blinded to clinical and exposure variables.

Every case was matched with one control without HCC (wo-HCC) in a 1:1 ratio according to age, gender, etiology of liver disease, Child Pugh score and presence of clinically significant portal hypertension. Exclusion of HCC in all controls was done with either CT or MRI scans during the screening period. Selection process of cases and controls was prior to evaluation of microbiome on stool samples, and was blinded from results of gut microbiome.

Healthy controls

In addition, we studied a gut microbiome dataset of a total of 25 matching healthy controls. These subjects were included in a previous epidemiological microbiome research from Rosario, Argentina [22]. This dataset was validated using the Illumina MiSeq technology as described below exclusively for this work.

Exposure variables: definition and meassurement.

The following measurements were recorded at baseline in each subject enrolled:

. *Demographic data:* age, weight and height, concomitant medications including use of lactulose, rifaximin or norfloxacin.

. *Laboratory* including red and white blood cells count, platelets, liver function tests, urea, creatinine, plasma electrolytes, prothrombin time, INR (International Normatized Ratio) and serum alpha-fetoprotein (AFP). These blood tests were measured in the same day or the day after fecal stool sample collection in all subjects blinded from clinical variables and microbiome results.

. *Clinical variables:* complete physical examination and past medical history, Child Pugh Score and presence or past history of clinically significant portal hypertension was evaluated during the screening period and revaluated at the same day of fecal sample stool collection. The latter included presence of ascites, hepatic encephalopathy (HE; West Haven grade criteria)[23] and presence of esophageal or gastric varices. The degree of ascites was classified as mild, moderate or severe according to ultrasonographic or physical examination.

. *Tumor characteristics:* Subjects with HCC diagnosis were staged according to Barcelona Clinic Liver Cancer staging (BCLC) [20,21,24] at study entry. Type of previous tumor treatment was registered: trans-arterial chemoembolization (TACE), radiofrequency ablation (RFA), percutaneous ethanol injection (PEI) and liver resection (LR).

Fecal stool samples collection, DNA extraction and sequencing.

Fecal samples were obtained noninvasively either at home or in the hospital, in a plastic collection kit at any time during the day. All samples were stored at -70°C. Samples obtained at patient's home, were maintained for 24hs at 4 ºC, until they were taken to the hospital and stored at -70 ºC.

Each fecal sample was aliquoted for final processing in Heritas laboratory in Rosario, Argentina. Total DNA extraction from stool samples (about 200 mg) was performed using QIAmp DNA Stool Mini Kit following manufacters's instructions. The 16S rRNA V3-V4 hypervariable region was firstly amplified using PCR method (20 cycles) and then a second reading for sample identification (6 cycles). Amplicons were cleaned using Ampure DNA capture beads (Argencourt-Beckman Coulter, Inc.) and quantified using Quanti-iTTM PicoGreen® DNA Assay Kit (Invitrogen Molecular Probes, Inc., Eugene, OR, USA) with the standard protocol (high range curve – half area plate) and pooled in molar concentrations before sequencing on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA) using 2x300 cycles PE v3 chemistry. In each procedure 3 measurements were made to avoid information bias. The operators of this measurement were blinded to any clinical variables, including information regarding if a subject was either a case or a control.

Sequence data pre-processing, classification and taxonomic assignment

Amplicon sequencing produced 11639500 raw paired-end (PE) reads. We followed the 16S SOP described in Microbiome Helper [27]. FastQC (v0.11.5) was used to analyze the raw data quality of PE reads. Paired-end reads were stitched together using PEAR (v0.9.10). Stitched reads were filtered by quality and length, using a quality score cut-off of Q30 (phred quality score) over 90% of bases and a minimum length of 400bp. Concatenated and filtered fastq sequences were converted to fasta format and we removed sequences that contain 'N'. Potential chimeras were checked using the UCHIME algorithm, and then the chimeric sequences were removed.

The remaining sequences were clustered to operational taxonomic units (OTUs) at 97% similarity level with a open reference strategy implemented in QIIME (v1.91),

using SortMeRNA for the reference picking against the SILVA (v119) database and SUMACLUST for de novo OTU picking.

Study end-points

The objective of this study was to compare gut microbiome profile in patients with cirrhosis with and without HCC, in order to identify a specific profile of microbiome potentially associated with the risk of developing liver cancer.

Potential biases and confounding variables

In order to anticipate and avoid selection bias regarding HCC-cases and wo-HCC, subjects were followed and treated under the same standards of care and were selected from a cohort of patients prospectively followed at Austral University Hospital by investigators who were blinded to gut microbiome results. Acceptances and declines to participate in this study were recorded. Selection bias was avoided, excluding HCC diagnosis with a CT or MRI scan in wo-HCC controls.

Statistical analysis

A statistical two-tailed value, α type I error or 5% (p value <0.05) was considered. Binary or dichotomous variables were expressed as frequencies or proportions and compared by Chi-square test or Fisher exact test, as appropriate. Dicrete and continuous numerical variables were expressed according to their distribution as mean (standard deviation) or medians (interquartile range) and were compared using Student t-test or Mann-Whitney U test, respectively. In case of multiple comparisons for continuous variables, a parametric ANOVA with Bonferroni correction was done. Clinical data statistical analyzes was done using STATA version 10.1.

Bioinformatics analysis of the data was done using a custom QIIME pipeline and Phyloseq. OTU table was rarefied at 12506 sequences per sample for alpha and beta diversity analyses. To provide alpha diversity metrics, we calculated observed species, Chao1 and Shannon's diversity index. To evaluate beta diversity among cases, controls and healthy individuals UniFrac (weighted and unweighted) distances and Bray Curtis similarity were used. The UniFrac and Bray Curtis measures were represented by two dimensional principal coordinates analysis (PCoA) plots. The R package phyloseq (https://github.com/joey711/phyloseq) was used to import and plot data, and the package DESeq2 was used to perform differential abundance estimates. The evaluation of beta diversity between cases (HCC) and controls (wo HCC) were also determined using a principal fitted components for dimension reduction in regression as it was used to analyze microbiome data [25].

Ethical considerations.

This study protocol has been developed according to national standards for ethical, legal and regulatory requirements established in the 6677/10 ANMAT disposition, international ethical standards according the 2008 Helsinki Declaration and its amendments from the Nuremberg code, universal declaration on the human genome and human rights adopted by the General Conference of UNESCO 1997, as well as GCP standards ("Good Clinical Practice").

Results.

Participating Patients characteristics

From a total of 407 patients who were prospectively followed and entered in the pre-screening study period, 72 patients with HCC and 190 without HCC were selected as

potential cases and controls. Overall, 25 cases and 25 controls (wo-HCC) were well matched according to those variables previously described and were included in the final assessement of gut microbiome (Figure 1). A subgroup of 25 healthy individuals were also enrolled, matched according to gender in a 1:1 ratio [26]. Baseline patient characteristics are shown on Table 1. Considering severity of liver disease, most of the patients were Child Pugh A (74%), only 10% had ascites and 8% had presence of HE.

Cases and controls: Clinical and tumor variables.

In patients with HCC the mean number of intrahepatic nodules were 2.2 ± 1.8, with a major nodule tumor diameter of 3.8 ± 1.6 cm. Considering BCLC stages, 2 patients were stage 0, 12 patients were stage A, 5 were in stage B and 6 in stage C-D. Vascular invasion was observed in 3 patients and extrahepatic spread in 4. Previous HCC treatments consisted of RFA in 1 patient, LR in 3, TACE in 17 and Sorafenib in 3 patients.

Table 2 shows baseline clinical and laboratory variables comparing cases and controls. There were no significant differences in any clinical variable. The use of rifaximin, norfloxacin or lactulose was not significantly different between cases and controls.

Alpha and beta diversity in the gut microbiome of Cases, Controls and Healthy subjects

Cases and controls (wo-HCC) datasets of the gut microbiome were compared to a gut microbiome dataset of healthy subjects from the city of Rosario (Argentina). The alpha diversity index indicated that healthy controls showed the most diverse dataset

of gut microbiome compared to cases and controls (Figure 2). On the contrary, wo-HCC dataset consistently and significantly showed the less diverse dataset of gut microbiome. The HCC dataset was less diverse than healthy controls but still more diverse than the wo-HCC dataset.

The beta diversity analysis performed using a log-data transformation in a Bray-Curtis PCoA showed that the cases and wo-HCC groups were clearly separated from the healthy control population (Figure 3A). To gain in-depth insight of cases and wo-HCC group, we performed a principal fitted components analysis with dimension reduction that showed a more precise and clear separation of the gut microbiome datasets between groups (Figure 3B). However, there were still a group of patients with no difference in beta diversity analysis of gut microbiome between cases and controls.

Taxonomic distribution analysis of the gut microbiome

Taxonomic abundance at Phylum, Family and Genus levels were investigated for cases, wo-HCC and healthy groups. Differences in the relative abundance of taxa were evident at the levels of Family and Genus among the three groups. Of note is the expansion of Bacteroidaceae and reduction of Prevotellaceae in the HCC group that, in turn, is reflected at the genus level in Bacteriodes and Prevotella. Consequently, the bacteriodes/prevotella ratio is greater in HCC than in the two other groups (Figure 4 A-C).

To further investigate which taxa is accounting for the observed differences in alpha and beta diversity of the gut microbiome in the HCC group, we performed a differential abundance analysis using phyloseq and DEseq2 (Figure 5). Several genera showed interesting correlations such as Fusobacterium, Prevotella, Streptoccocus,

S24-7 (Phylum Bacteroidetes) and an unknown genus (phylum Firmicutes, family Leuconostocaceae), all of which were decreased 2 to 5-fold in HCC group. On the other hand, Haemophilus, Eggerthella, Bifidobacterium, Butyricimonas, Christensella, Odoribacter, an unknown genus (phylum Tenericutes) and an unknown genus (phylum Firmicutes, family Erysipelotrichaceae) were all elevated by 2 to 3-fold in HCC group (Figure 4). Each genus in Figure 4 is the result of the mean abundance of several OTUs contained in that genus and only those with significant differential abundances with an adjusted *P* value of 0.05 are shown.

Individual OTUs within some of the genera showed greater o lower values of fold change in the HCC group. For instance, several OTUs in Prevotella genus decreased between 3 to 6-fold but other Prevotella OTUs increased 1.5 to 3-fold. The mean fold change for genus Prevotella indicated a significant (*P* value=0.05) 3-fold decreased as shown in Figure 4. A full panorama involving the complete dataset of OTUs analyzed with significant changes is observed in supplementary Figure 1 and supplementary Table.

Interestingly, all the changes in abundance in Figure 4 correlated with an increase in the predicted metabolism of NOD-like receptor (NLR) signaling pathway in the HCC group (Supplemenatary Figure 2). The NLR signaling pathway was previously reported to be involved in inflammatory and autoinflammatory processes [14].

Discussion

To the best of our knowledge, this is the first observational study to identify a specific gut microbiome among patients with cirrhosis with and without HCC. Patients with HCC had lower quantities of genus Prevotella, Fusobacterium and family Leuconostocaccaceae among others and an elevated proportion of genus

Bifidobacterium, Eggerthella, Haemophilus, family Erysipelotrichaceae and phylum Tenericutes when compared to controls without HCC. This pattern has been associated with an inflammatory milieu with increased activation of NLR signalling pathways.

Interestingly, in our study the HCC group showed a more diverse gut microbiome than the wo-HCC group but still less diverse than a healthy population control. This observation could be relevant in the context of disease development and progression suggesting an expansion of certain families or genera of microbes in the tumor microenvironment with an inflammatory milieu. These findings suggest a tumor specific expansion of microbiota [27].

It is interesting to note that some families in the phylum Firmicutes were either significantly decreased or elevated in HCC cases versus controls wo-HCC. Hepatocellular carcinoma is a pro-inflamatory cancer as previously mentioned [15]. We observed a 3-fold increased of Erysipelotrichaceae in HCC patients. This family of firmicutes has been implicated in inflammation related to gastrointestinal diseases and in tumor development such as colorectal cancer [28]. Besides, the relative abundance of Erysipelotrichi positively correlated with tumor necrosis factor alpha (TNF) levels in a patients with HIV infection [29]. Further evidence showed that the abundance of Erysipelotrichi were also positively associated with changes in fatty liver in female subjects who were placed on diets in which choline levels were manipulated [30].

On the other hand, family Leuconostocaceae, a main producer of acetate and lactate, was decreased by 5-fold in HCC cases versus controls wo-HCC, as also observed in ulcerative colitis [31]. It is important to mention that the two genera of Firmicutes could potentially serve as predictive biomarker of HCC, since in wo-HCC was decreased by 5-fold and in HCC was increased by 3-fold.

Several members of the gut microbiome were also found significantly decreased in HCC cases. For instance, the genus Fusobacterium was found 5-fold decreased in HCC versus wo-HCC. Elevated levels of Fusobacterium were found associated with tumorigenesis process in the gut and a proinflammatory microenvironment[32]. However, we found decreased levels in the HCC cases; showing that a specific micriobiome related cancer might be found in different tumors.

Bacteroides and Prevotella are major genus of the Bacteriodetes phylum present in the gut, but they are mutually excluded in the dominance as they are clearly competitors for the niche. Prevotella are usually found in non-western diets such as plant-rich fiber diets. However, prevotella was also linked to chronic inflammation processes. In our study, the ratio bacteriodes/prevotella was increased in HCC cases due to the significant decrease in the genus prevotella and no significant changes in the genus bacteroides. In fact, prevotella was decreased by at least 3-fold in HCC cases. This observation is under further investigation by our group.

Finally, the key question is whether these findings observed in this case-control study, will be repeated in a cohort study evaluating microbiome changes over time. Our group is in a second phase of research including a longitudinal microbiome measurement. This will allow us to clarify whether these preliminary observations explain a novel pathophysiological mechanism in the generation of HCC.

In our study, changes in abundance in HCC patients correlated with an increase in the predicted metabolism of NOD-like receptor (NLRs) signaling pathway. However, we are aware that abundant molecular functions are not necessarily provided by abundant species, highlighting the importance of a functional analysis for a community understanding [14]. NOD-like receptors are intracellular sensor with central roles in innate and adaptive immunity. Whereas the membrane-bound Toll-like receptors

(TLRs) survey the extracellular milieu, NLRs have emerged as key sensors of infection and stress in intracellular compartments [14]. These molecules act as sensors of microbial fragments inside the human cytosol of infected cells. Once activated, NLRs recruit NF-κβ and facilitate the production of interferon type I. These NLRs can regulate several aspects of the immune response.

In this sense, it has been previously observed that in cirrhosis, a "leaky" gut accounting for an impaired intestinal barrier function and bacterial overgrowth leads to development of main clinical events in cirrhosis. These bacterial products induce inflammation through activation of TLR and NLRs in the liver, inducing a inflammatory environment of cancer development [12,13].

We recognize that this study has limitations including first, that in case-control studies, several predictive factors might be biased. However, a strict revision of potentially selection and information bias was done. Second, the limited number of included patients is a main caveat. However, these preliminary observations should be evaluated in a longitudinal assessment of gut microbiota in cirrhotic patients with higher risk of HCC development.

In conclusion, we found several different genera and families in the gut microbiota that were decreased or elevated in HCC cases versus controls (wo-HCC). Patients with HCC had lower quantities of genus Prevotella, Fusobacterium and family Leuconostocaccaceae among others and an elevated proportion of genus Bifidobacterium, Eggerthella, Haemophilus, family Erysipelotrichaceae and phylum Tenericutes when compared to controls without HCC. This pattern was associated with inflammatory pathways. It would be interesting to explore the utility of these members as biomarkers of disease progression in a larger cohort study and eventually, in an intervention prevention trial.

Authorship statement.

All the authors approved the final version of the manuscript.

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FIGURE LEGENDS.

Figure 1. Inclusion and Exclusion Criteria flow chart.

Figure 2, Panel plots comparing alpha-diversity between cases (HCC), controls (wo-HCC) and healthy individuals.

Note: Healthy controls showed the most diverse dataset of gut microbiome. Patients with wo-HCC dataset consistently and significantly showed the less diverse dataset of gut microbiome, while cases (HCC) being less diverse than healthy controls, still more diverse than controls (wo-HCC) dataset.

Figure 3. Betha-diversity plots between cases (HCC), controls (wo-HCC) and healthy individuals (Panel A, Bray Curtis –log; Panel B, Principal Fitted Components Analysis).

Note: Cases and control wo-HCC groups were clearly separated from the healthy control population (Panel A). A more precise and clear separation of the gut microbiome datasets between groups is show non Panel B.

Figure 4. Taxnomic abundance at Phylum, Family and Genus levels between HCC, non-HCC patients and healthy individuals (Panels A-C).

Note: A bacteriodes/prevotella ratio was greater in HCC than in non-HCC controls and healthy controls.

Figure 5. Differential abundance analysis using (Phyloseq and DEseq2).

Note: Several genera showed interesting correlations such as Fusobacterium, Prevotella, Streptoccocus, S24-7 (Phylum Bacteroidetes) and an unknown genus

(phylum Firmicutes, family Leuconostocaceae), all of which were decreased 2 to 5-fold in HCC group.

Supplementary Figures, S1 and S2.

Note S1: Several OTUs in Prevotella genus decreased in HCC cases.

Note S2: Interestingly, all the changes in abundance in HCC patients correlated with an increase in the predicted metabolism of NOD-like receptor signaling pathway.

Figure 1.

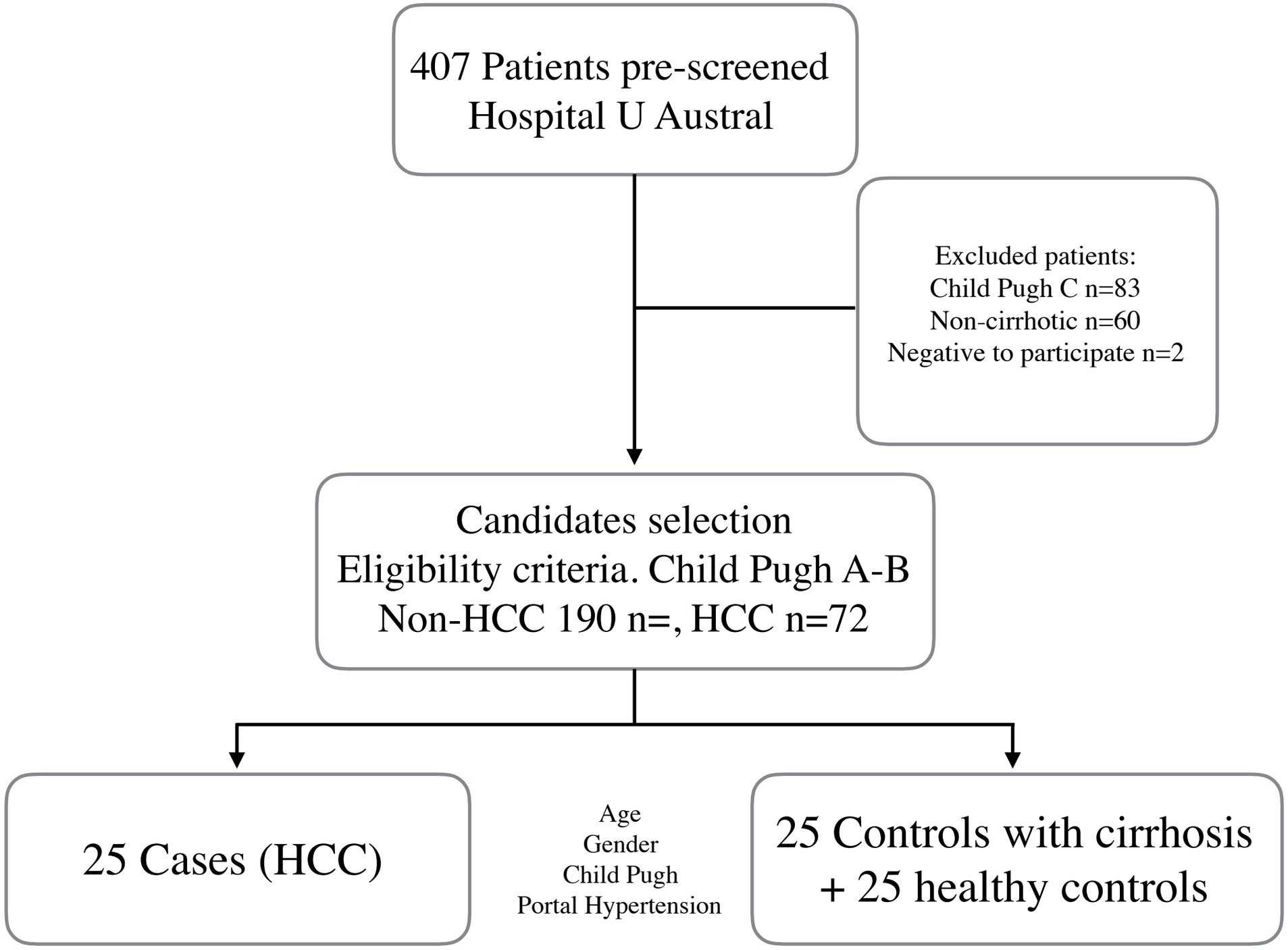

407 Patients pre-screened
Hospital U Austral
Excluded patients:
Child Pugh C n=83
Non-cirrhotic n=60
Negative to participate n=2
Candidates selection
Eligibility criteria. Child Pugh A-B
Non-HCC 190 n=, HCC n=72
25 Cases (HCC)
Age
Gender
Child Pugh
Portal Hypertension
25 Controls with cirrhosis
+ 25 healthy controls

Figure 2.

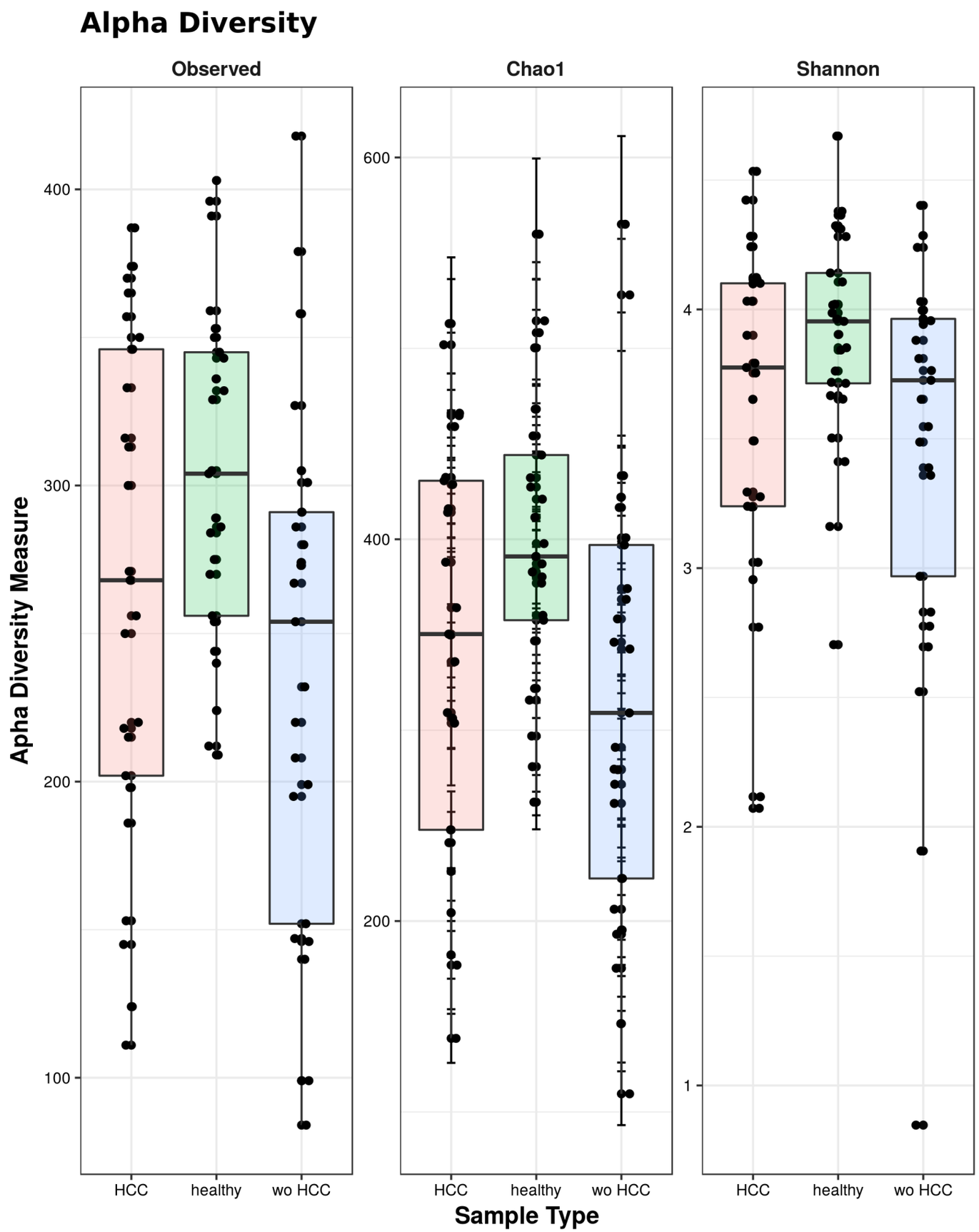
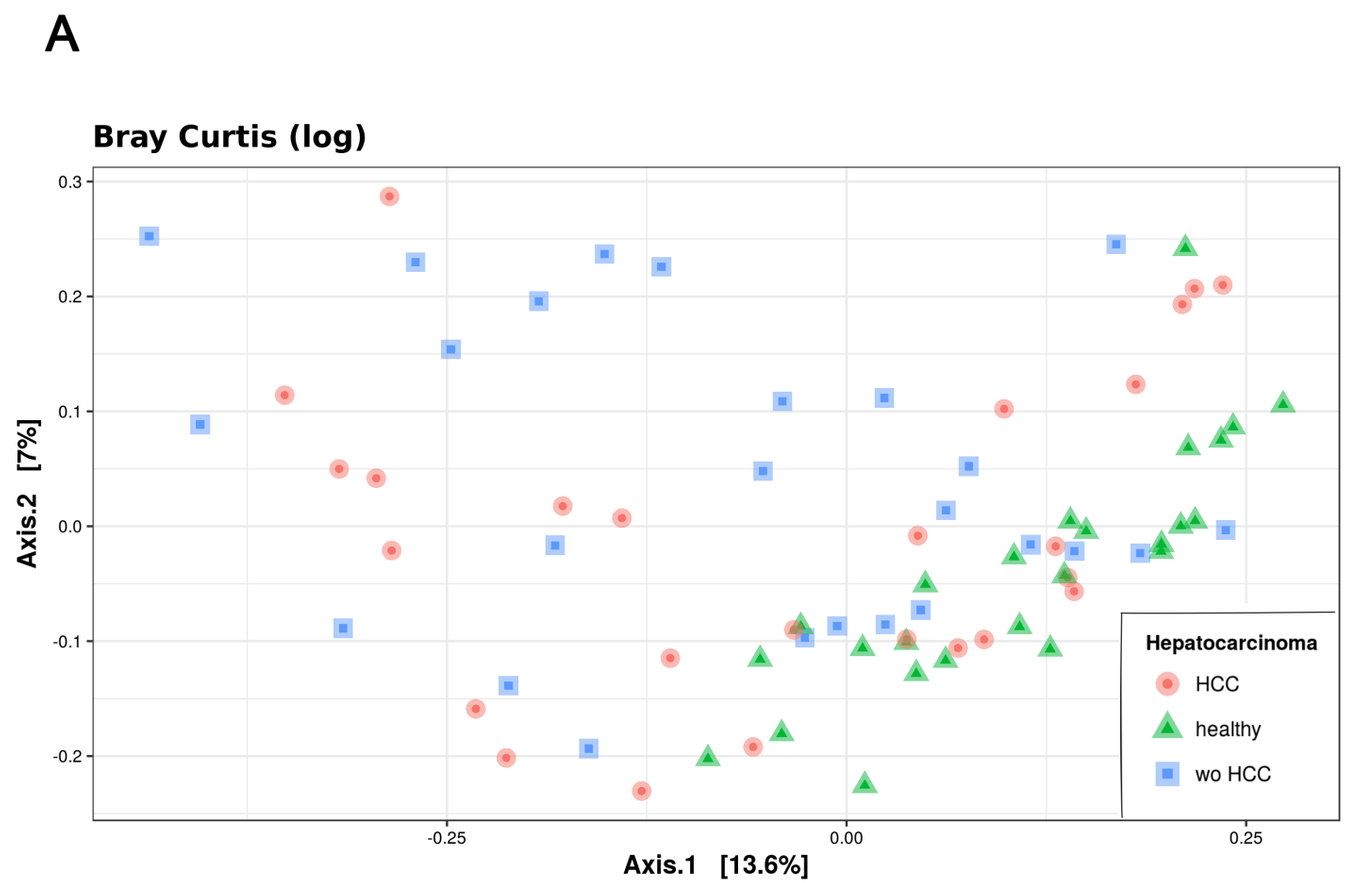

Alpha Diversity
Observed
Chao1
Shannon
Apha Diversity Measure
400
300
200
100
600
400
200
4
3
2
1
HCC
healthy
wo HCC
Sample Type

Figure 3.


A
Bray Curtis (log)
Axis.2 [7%]
Axis.1 [13.6%]
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.25
0.00
0.25
Hepatocarcinoma
HCC
healthy
wo HCC

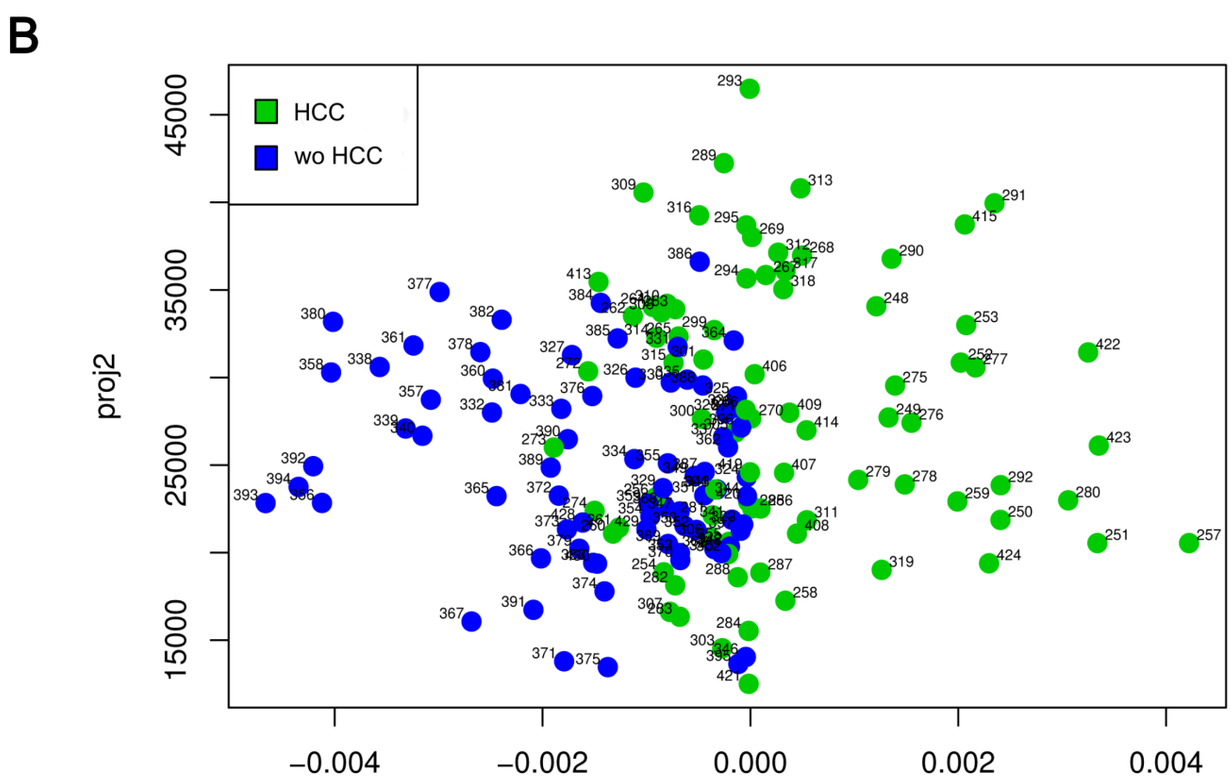

B
HCC
wo HCC
proj2
45000
35000
25000
15000
−0.004
−0.002
0.000
0.002
0.004

Figure 4.

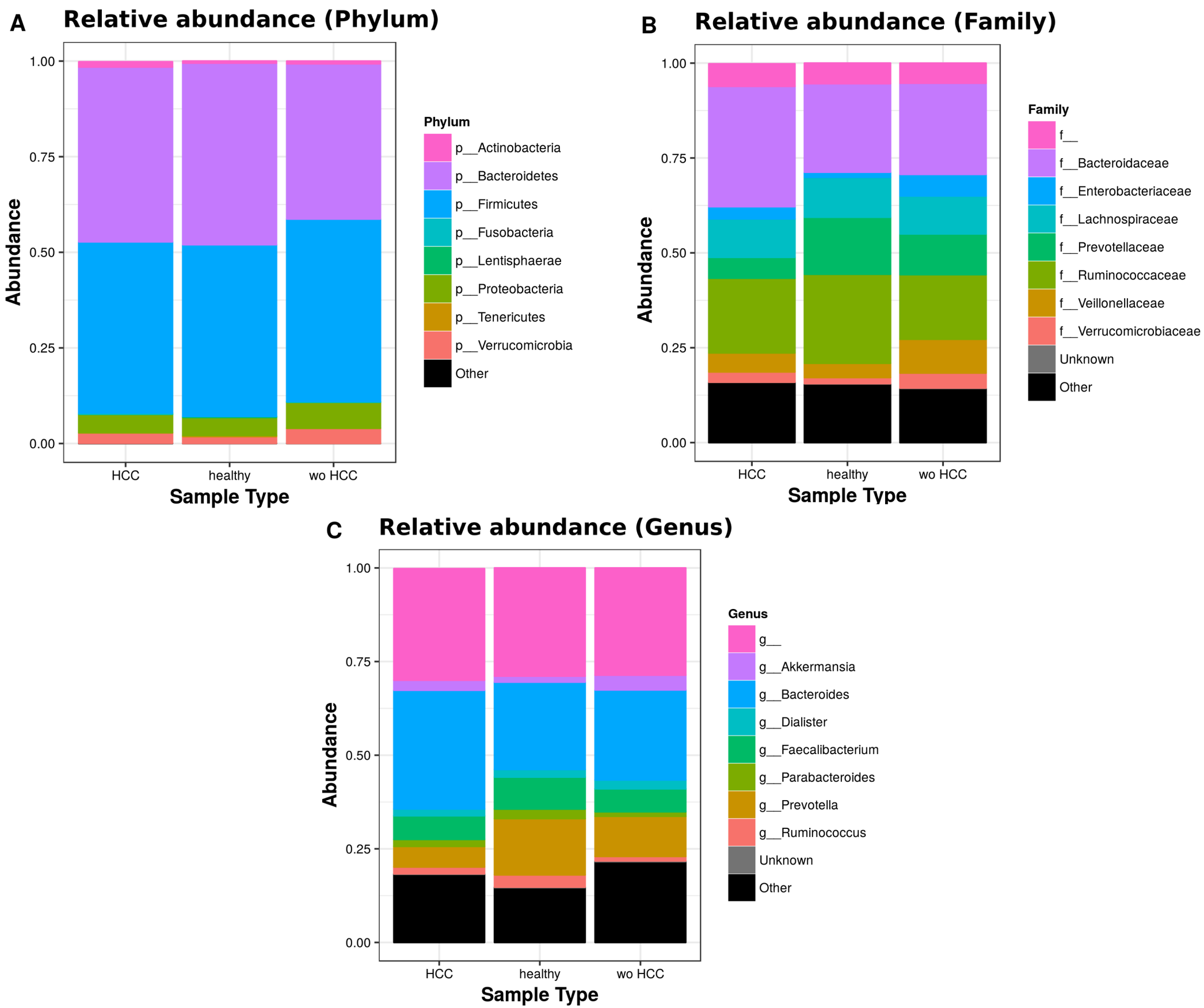
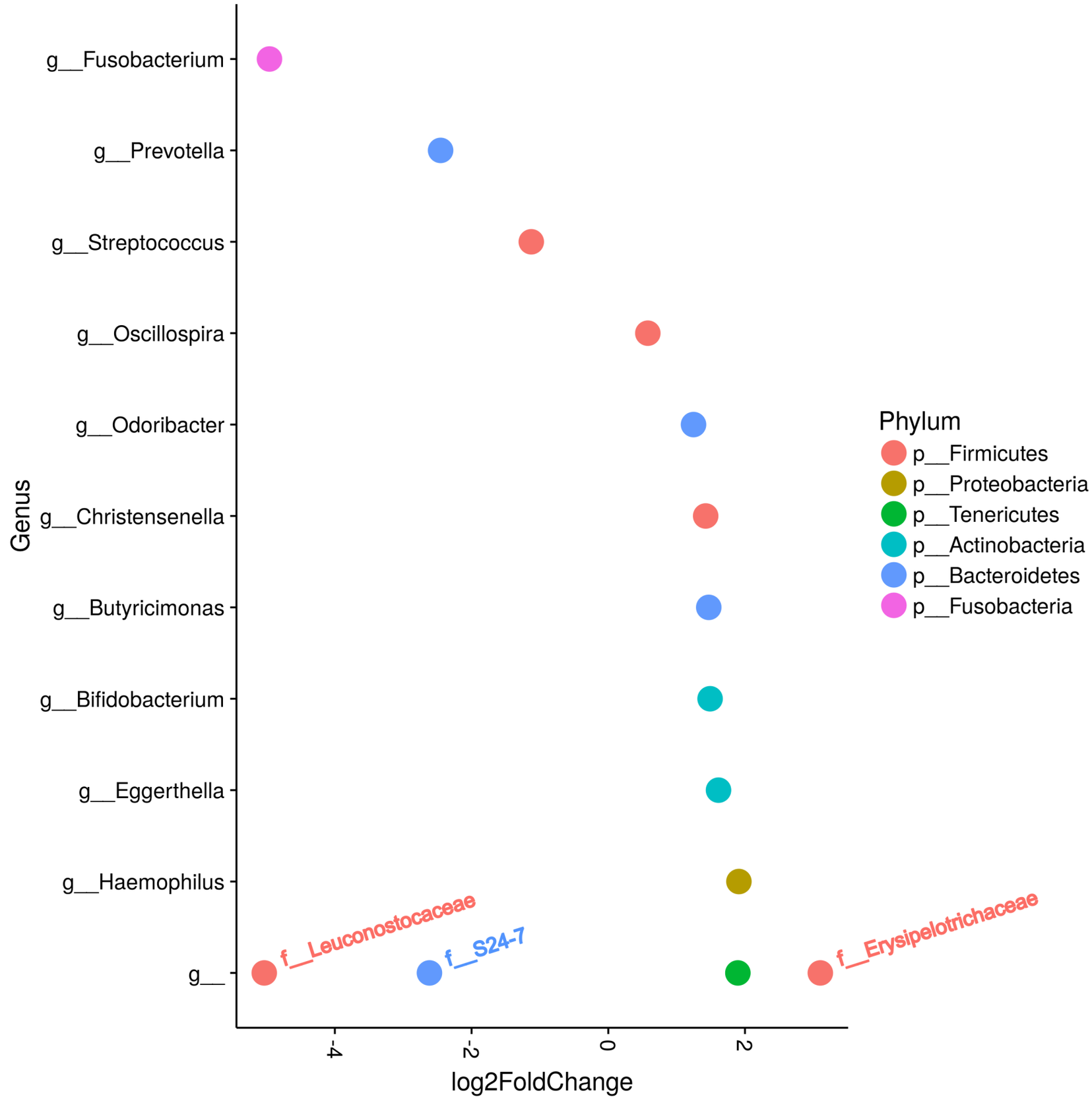
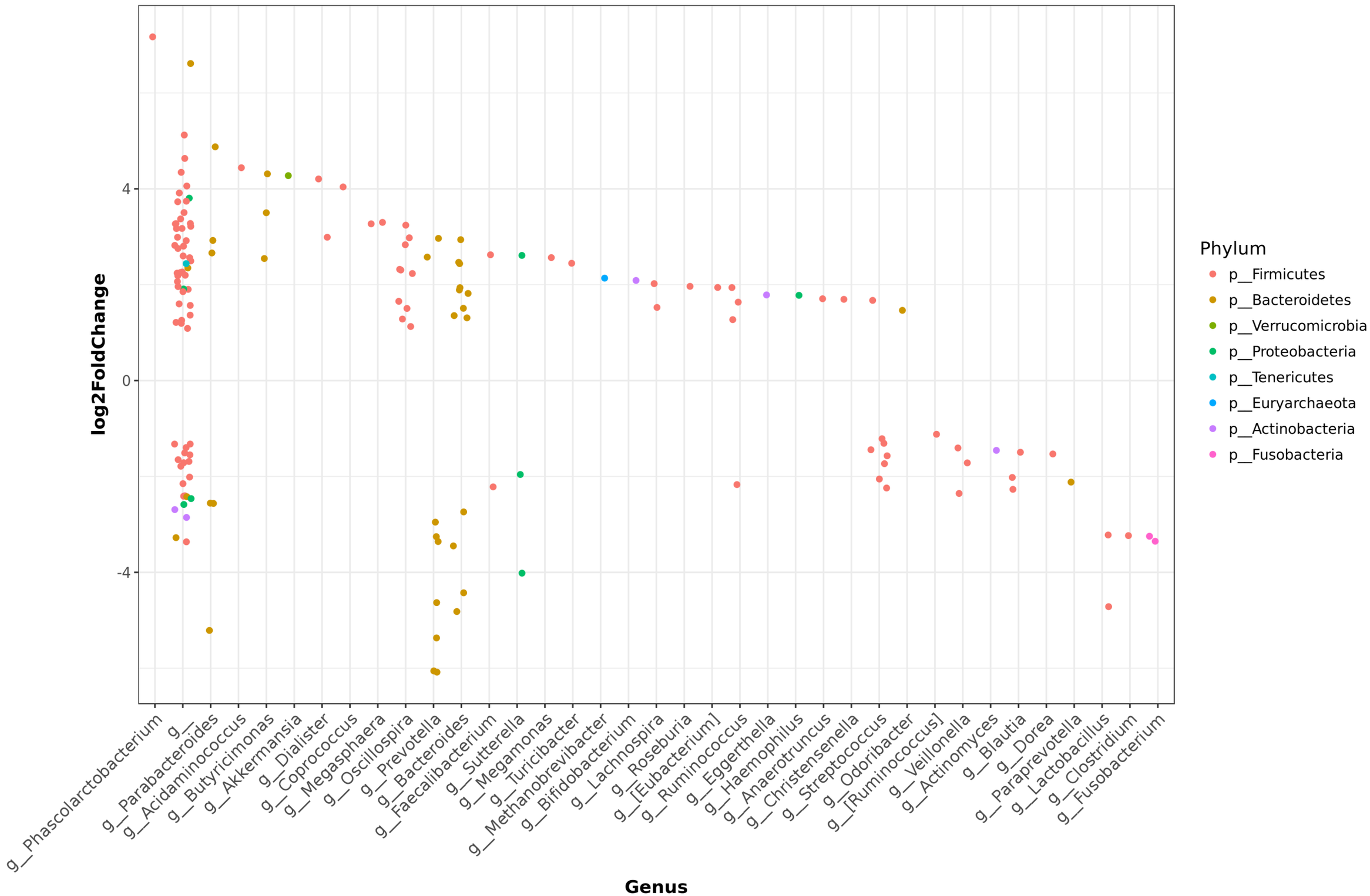

A
Relative abundance (Phylum)
Abundance
1.00
0.75
0.50
0.25
0.00
HCC
healthy
wo HCC
Sample Type
Phylum
p__Actinobacteria
p__Bacteroidetes
p__Firmicutes
p__Fusobacteria
p__Lentisphaerae
p__Proteobacteria
p__Tenericutes
p__Verrucomicrobia
Other
B
Relative abundance (Family)
Abundance
1.00
0.75
0.50
0.25
0.00
HCC
healthy
wo HCC
Sample Type
Family
f__
f__Bacteroidaceae
f__Enterobacteriaceae
f__Lachnospiraceae
f__Prevotellaceae
f__Ruminococcaceae
f__Veillonellaceae
f__Verrucomicrobiaceae
Unknown
Other
C
Relative abundance (Genus)
Abundance
1.00
0.75
0.50
0.25
0.00
HCC
healthy
wo HCC
Sample Type
Genus
g__
g__Akkermansia
g__Bacteroides
g__Dialister
g__Faecalibacterium
g__Parabacteroides
g__Prevotella
g__Ruminococcus
Unknown
Other

Figure 5.


g__Fusobacterium
g__Prevotella
g__Streptococcus
g__Oscillospira
g__Odoribacter
g__Christensenella
g__Butyricimonas
g__Bifidobacterium
g__Eggerthella
g__Haemophilus
g__
Genus
f__Leuconostocaceae
f__S24-7
f__Erysipelotrichaceae
-4
-2
0
2
log2FoldChange
Phylum
p__Firmicutes
p__Proteobacteria
p__Tenericutes
p__Actinobacteria
p__Bacteroidetes
p__Fusobacteria

Supplementary Figure 1.



Supplementary Figure 2.

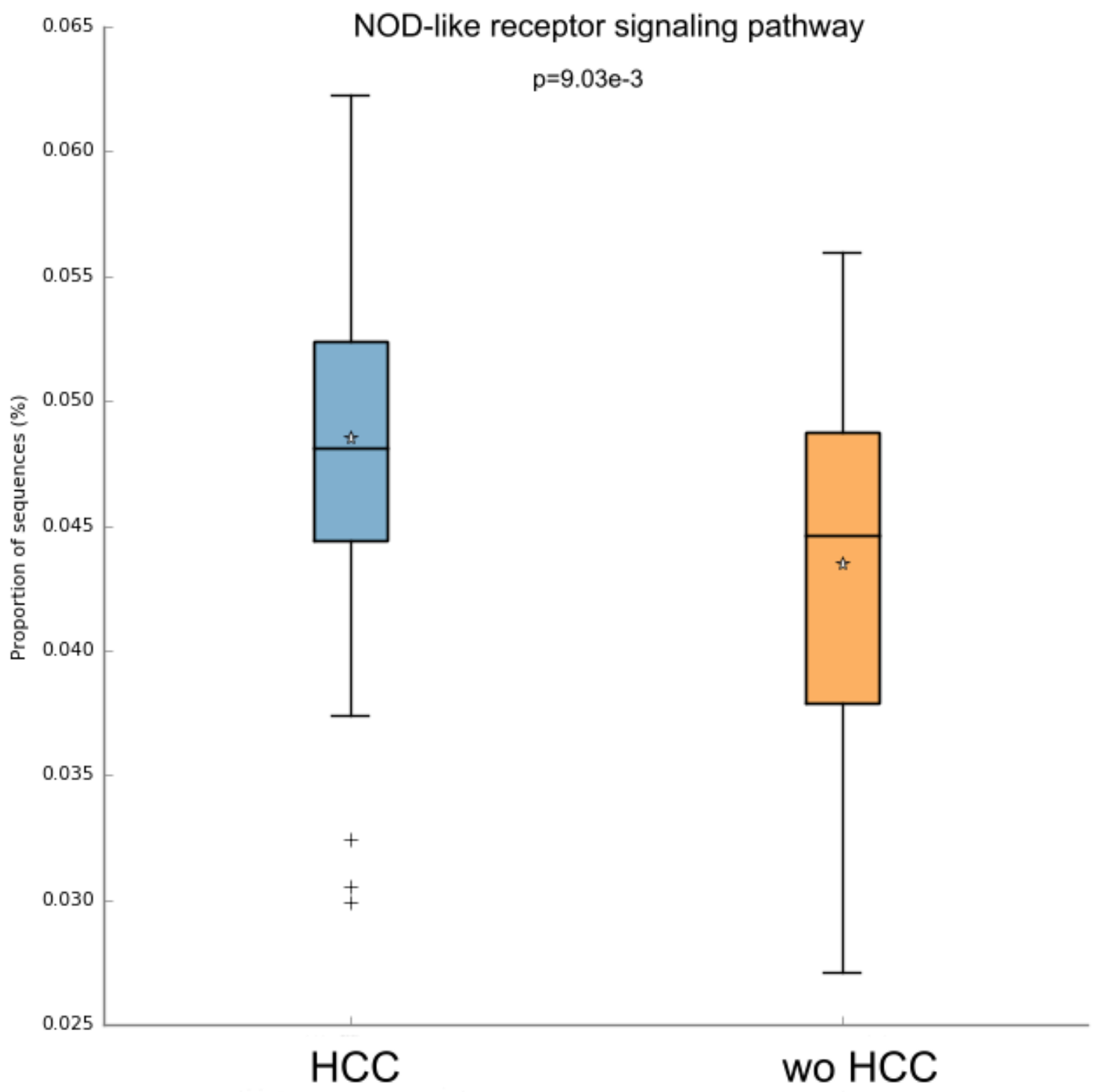


Table 1. Patients' baseline characteristics.

VARIABLE	*Values*
Age, years (± SD)	64 ± 8
Male gender, n (%)	44 (88.0)
BMI, Kg/m^2	27.9 ± 4.5
Child Pugh A/B, n (%)	37 (74.0)/13 (26.0)
Etiology of liver disease, n (%)	
Hepatitis C virus	13 (26.0)
Alcohol	11 (22.0)
NASH	10 (20.0)
Cryptogenic	8 (16.0)
Hepatitis B virus	4 (8.0)
Autoimmune	2 (4.0)
Hemochromatosis	2 (4.0)
Ascites, n (%)	
No	45 (90.0)
Mild	2 (4.0)
Moderate-severe	3 (6.0)
Encephalopathy, n (%)	
No	46 (92.0)
Grade I-II	4 (8.0)
Grade III-IV	-
Esophageal varices, n (%)	41 (82.0)
ECOG 0-2/3-4, n (%)	50 (100)/ -

Abbreviations: NASH, non-alcoholic steatohepatitis. BMI, Body Mass Index.

Table 2. Baseline clinical and laboratory variables among cases and controls.

VARIABLE	*Cases (HCC)* *n= 25 (50%)*	*Controls (wo-HCC)* *n= 25 (50%)*	*P*
Age, years (± SD)	64 ± 9	63 ± 7	0.53
Male gender, n (%)	22 (88.0)	22 (88.0)	1.0
Diabetes mellitus, n (%)	14 (.56.0)	8 (32.0)	0.17
BMI, Kg/m^2	27.9 ± 5.4	28.1 ± 3.6	0.87
Child Pugh A/B, n (%)	17 (68.0)/8 (32.0)	20 (80.0)/5 (20.0)	0.93
MELD	6.7 ± 0.4	6.6 ± 0.5	0.76
Etiology of liver disease, n (%)			
Hepatitis C virus	6 (24.0)	7 (28.0)	
Alcohol	6 (24.0)	5 (20.0)	
NASH	5 (20.0)	5 (20.0)	
Cryptogenic	4 (16.0)	4 (16.0)	
Hepatitis B virus	2 (8.0)	2 (8.0)	
Autoimmune	1 (4.0)	1 (4.0)	
Hemochromatosis	1 (4.0)	1 (4.0)	
Ascites, n (%)			
No	22 (88.0)	23 (92.0)	0.31
Mild	2 (8.0)	0 (0.0)	
Moderate-severe	1 (4.0)	2 (8.0)	
Encephalopathy, n (%)			
No	23 (92.0)	23 (92.0)	1.0
Grade I-II	2 (8.0)	2 (8.0)	
Grade III-IV	-	-	
Portal hypertension, n (%)	20 (80.0)	23 (92.0)	0.22
History of decompensation, n (%)	9 (36.0)	14 (56.0)	0.15
Ascites	3 (12.0)	6 (24)	
Variceal Hemorrhage	2 (8.0)	1 (4.0)	
Encephalopathy	0 (0.0)	1 (4.0)	
Rifaximin/Norfloxacin, n (%)	3 (12.0)	5 (20.0)	0.44
Lactulose, n (%)	2 (8.0)	6 (24.0)	0.12
Hemoglobin, gr/dL (± SD)	13.2 ± 2.4	13.0 ± 2.2	0.81
Platelets /mm3 (± SD)	116,920 ± 57,753	124,640 ± 53,489	0.62

Creatinine, mg/dL (± SD)	0.88 ± 0.20	0.92 ± 0.28	0.55
Bilirrubin, mg/dL (± SD)	1.48 ± 0.98	1.26 ± 0.86	0.41
INR	1.33 ± 0.25	1.34 ± 0.30	0.93
AST, IU/L (± SD)	55 ± 46	43 ± 29	0.28
ALT, IU/L (± SD)	44 ± 32	38 ± 25	0.42
AP, IU/L (± SD)	150 ± 99	140 ± 73	0.68
γGT, IU/L (± SD)	192 ± 160	115 ± 91	0.07
AFP, ng/mL, median (IQR)	9.4 (3.2-218.0)	3.2 (1.6-5.5)	0.007

Abbreviations: AFP, alpha-fetoprotein. ALT, Alanine aminotransferase. AP, Alkaline Phosphatase. AST, Aspartate aminotransferase. γGT, Gamma glutamil transpeptidase. INR, International Normatized Ratio. NASH, non-alcoholic steatohepatitis.