Muntsa Rocafort
Irsicaixa – AIDS Research Lab
Hospital Germans Trias i Pujol
Ctra. de Canyet s/n, 08916
Badalona, Catalonia, Spain
mrocafort@irsicaixa.es

This study is supported by the University and Research Secretary - Department of Economy and Knowledge of the Government of Catalonia and the European Social Fund (Ref. 2015 FI_B 00184).

Evolution of The Gut Microbiome Following Primary HIV-1 infection

Muntsa Rocafort[1,2], Marc Noguera-Julian[1,2,3], Javier Rivera[1,3], Lucía Pastor[1,4,5,6], Yolanda Guillén[1], Jost Langhorst[7], Mariona Parera[1], Inacio Mandomando[4,5], Jorge Carrillo[1,4], Bonaventura Clotet[1,2,6], Julià Blanco[1,3,6], Denise Naniche[4,5], Roger Paredes[1,2,6]

[1]*irsiCaixa AIDS Research Institute, Ctra de Canyet s/n, Badalona, Catalonia, Spain;* [2]*Universitat Autònoma de Barcelona, Bellaterra, Catalonia, Spain;* [3]*Universitat de Vic-Universitat Central de Catalunya, Vic, Catalonia, Spain;* [4]*Barcelona Institute for Global Health (ISGLOBAL), Barcelona, Catalonia, Spain;* [5]*Centro de Investigação em Saúde da Manhiça (CISM), Maputo, Mozambique;* [6]*Institut Germans Trias i Pujol (IGTP), Hospital Germans Trias i Pujol, Badalona, Catalonia, Spain;* [7]*University of Duisburg-Essen, Kliniken Essen-Mitte, Internal Medicine V, Am Deimelsberg, Essen, Germany*

Poster Number CROI2016 : **260**

This study has been co-financiated by the Fondo Europeo de Desarrollo Regional and the Bill & Melinda Gates foundation

"Una manera de hacer Europa"

BACKGROUND

The early events following acute HIV-1 infection might be critical to shape the long-term interaction between the human gut microbiome and the host. In previous studies in non-human primates, SIV infection was followed by expansion of the enteric virome but had a limited impact on the gut bacteriome. Human data are scarce.

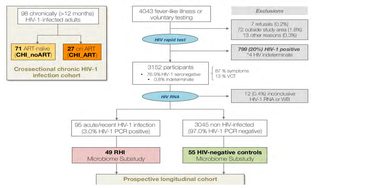
MATERIALS AND METHODS

STUDY DESIGN

We prospectively evaluated 103 subjects who attended the outpatient clinic of the Manhiça District Hospital in Mozambique with fever, and were either diagnosed with primary HIV-1 infection (PHI, n=49) or were HIV-1-negative (n=54) (baseline). Longitudinal fecal samples were collected over the first year starting at month 1, and were compared with single fecal samples from 98 chronically HIV-1-infected (CHI) subjects, 27 on antiretroviral treatment (ART) and 71 ART-naïve.







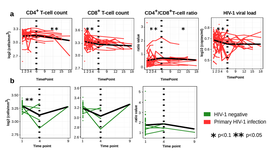
METADATA

Demographic and clinical data were collected in a specific questionnaire which included the study group to which the patient belonged (PHI, NEG, CHI_ART and CHI_noART), the time point of follow-up in months, the participant's gender, age, weight and height, CD4+ and CD8+ T-cell counts, the CD4+/CD8+ ratio, HIV-1 RNA levels, and hemogram; the body temperature, whether fever was reported or not within the previous 24h of sample collection, whether the patient reported or not diarrhea the week before sample collection and its severity, as well as information on pregnancy and usage of ART and antibiotic if needed.

The dataset also included information on a malaria test performed the day of sample collection, whether the participant reported malaria within the previous month of sample collection, the presence of occult blood in feces, and the result of several microbiological tests in feces, including *Giardia spp., Cryptosporidium spp., Entamoeba spp., Clostridium difficile* toxin as well as serologies for *Hepatitis B* and *C, syphilis* and *Strongyloides stercoralis*.

	Recently HIV-infected	Chronically HIV-infected, no ART	Chronically HIV-infected, with ART	HIV-Negative	P value PHI vs neg	P value Overall
N = 202	**49**	**71**	**27**	**55**		
Female Gender	32 (65%)	52 (73%)	15 (55%)	43 (78%)	0.189	0.154
Age (years)	26 (20; 30)	35 (29; 45.5)	42 (36.5; 46.5)	25 (21; 37)	0.210	**<0.001**
Weight (kg)	55 (49; 62)	63 (53.5; 68.5)	61 (60; 68.5)	58.5 (52; 67)	0.093	**0.007**
Height (cm)	160 (155; 169.7)	160 (154.2; 164)	164 (157.5; 167.5)	163.9 (161; 170.8)	0.192	**0.025**
BMI	21 (19.1; 23.5)	23.7 (21; 27.6)	23.3 (21.5; 25)	22.2 (18.5; 25)	0.637	**<0.001**
Pregnancy	3 (6.1%)	9	0	7 (12.7%)	0.730	**0.003**
CD4+ T cell /mm3	572 (409; 677)	533 (430.5; 726)	460 (366; 577.5)	928 (741; 1142.8)	**<0.001**	**<0.001**
CD8+ T cell /mm3	1229 (740; 1683)	988 (783.5; 1304)	834 (653.5; 1150)	591 (387.5; 689.7)	**<0.001**	**<0.001**
CD4+/CD8+	0.42 (0.23; 0.70)	0.56 (0.38; 0.79)	0.52 (0.33; 0.88)	1.65 (1.21; 2.24)	**<0.001**	**<0.001**
HIV-1 RNA @ t1 (copies/mL)	1082220 (318986; 2738500)	33400 (7288; 105200)	75 (75; 272.5)	-	-	**<0.001**
HIV-1 RNA @ screening (copies/mL)	2752700 (179220; 15078000)	-	-	-	-	-
Time with HIV, days	-	1344 (977; 1737)	1814 (1463; 2303)	-	-	**0.018**
Time on ART, days	-	-	1257 (678; 1645)	-	-	-
0 – 29.25 days	24 (49%)	-	-	-	-	-
29.25 - 29.30 days	7 (14.2%)	-	-	-	-	-
Delay between stage 1 - 30-100 days	7 (14.2%)	-	-	-	-	-
30 - >100 days	11 (22.4%)	-	-	-	-	-

White blood cells	5.8 (4.4; 6.7)	5.2 (4.2; 6.4)	3.7 (3.2; 5)	5.2 (4.4; 6.5)	0.413	**<0.001**
Hemoglobin	12.1 (10.9; 13.1)	11.8 (10.4; 12.7)	11.4 (10.8; 12.7)	12.3 (11.6; 13.2)	0.409	0.144
Hematocrite	36.9 (33.5; 39.8)	35.6 (32.7; 38.5)	34.6 (33; 38)	37.7 (34.7; 40.1)	0.320	**0.044**
Platelets	184.5 (130.7; 290.7)	205 (174.5; 258)	192 (173.5; 258)	226 (185; 265.5)	**0.004**	**0.028**
Hepatitis B	4 (8.1%)	2 (2.8%)	3 (11.1%)	1 (1.8%)	0.173	0.125
Syphilis	2 (4.1%)	0	1 (3.7%)	1 (1.8%)	0.588	0.088
Diarrhea, previous week	7 (14.3%)	3 (4.2%)	1 (3.7%)	4 (7.3%)	0.343	0.214
Temperature (ºC)	36.3 (36.2; 36.5)	36.4 (36.2; 36.6)	36.4 (36.2; 36.6)	36.2 (36.1; 36.5)	0.956	0.509
Fever, previous 24h	7 (14.3%)	3 (4.2%)	1 (3.7%)	3 (5.4%)	0.186	0.186
Any enteric pathogen	9 (18.3%)	12 (16.9%)	6 (22.2%)	11 (20%)	1	0.889
Blastsy hominis	2 (4.1%)	2 (2.8%)	0	1 (1.81%)	0.600	0.864
Entamoeba hystolitica	2 (4.1%)	6 (8.4%)	1 (3.7%)	2 (3.6%)	1	0.733
Giardia spp	2 (4.1%)	2 (2.8%)	0	5 (9.1%)	0.438	0.236
Cryptosporidium spp	1 (2%)	2 (2.8%)	0	0	1	0.636
Clostridium dif. cde	2 (4.1%)	1 (1.4%)	1 (3.7%)	1 (1.8%)	0.602	0.458
Strongyloides	1 (2.0%)	0	0	1 (1.8%)	1	0.636
Malaria, prev. month	7 (14.3%)	1 (1.4%)	0	9 (16.4%)	1	**<0.001**
Current Malaria test	2 (4.1%)	1 (1.4%)	0	0	-	0.071
Malaria test severity	3 (1.5; 3)	4 (4; 4)	NA	NA	-	0.071



FECAL MICROBIOME TESTING

DNA and RNA were isolated from frozen fecal samples. The fecal bacteriome was characterized by amplifying the variable region V3-V4 from bacterial universal molecular marker 16S rRNA gene and further sequencing with MiSeqTM technology (300 paired-end reads). Generated sequencing data served as input to QIIME to taxonomically characterize microbial commmunities using GreenGenes 13.8 reference database.

Metagenomic Shotgun sequencing (MGS) was also performed at month 1, 4 and 9 samples in 13 subjects with PHI and 8 HIV-negative individuals. Whole fecal DNA was fragmented with the Nextera-XT® Illumina kit generating libraries of approximately 300 basepair insert sizes and sequenced using Illumina Hi-Seq technology. Quality filtered sequences were mapped against Integrated Gene Catalog (IGC) and KEGG database for a functional profiling of the samples.

VIROME TESTING

Presence of fecal Adenovirus (ADV), Cytomegalovirus (CMV), Enterovirus (ETV), and Human Herpes Viruses (HHV) 6A, 6B and 8 was evaluated by commercial real time PCR kits on already extracted frozen DNA and RNA aliquotes.



STATISTICAL ANALYSIS

Ecological analyses on the OTU table for the 16S rRNA data were performed using R/Vegan and R/Phyloseq packages. Statistical analyses for richness estimators and diversity indices among groups were done with Wilcoxon-Rank sum test and Kruskal-Wallis test as corresponded. Longitudinal evolution of ecological parameters were analyzed using Linear Mixed Models (LMM). Similar tests were used to assess relative abundance of bacterial genera and gene richness among groups and over time.

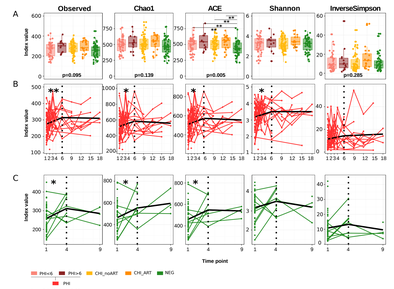
Virome expansion was assessed by presence/absense of viral DNA/RNA in feces. Comparison of number of individuals with viruses- in feces among groups at study entry was performed using Fisher's exact statistical test.

RESULTS AND DISCUSSION

16S rRNA gene sequencing

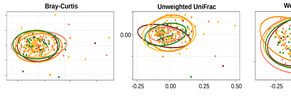
1) Hyper-acute dysbiosis linked to HIV-1 infection impacts on gut bacterial taxonomic richness and diversity

A. Boxplots (median±IQR) show richness (Observed, Chao1 and ACE) and diversity (Shannon and Inverse Simpson) estimators. Inverse Simpson index is computed as 1/Simpson concentration index. Baseline dataset includes 46 PHI<6, 13 PHI>6, 61 CHI_noART, 21 CHI_ART and 52 HIV-1 negative.
B and **C.** Results of linear mixed models (LMM) assessing longitudinal behaviour of ecological indices in the NEG (B, n=64) and RHI (C, n=136) samples.

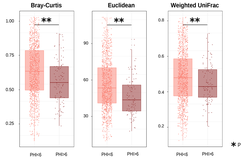


2) No overall bacterial genera composition is linked to HIV-1 infection but a significant reduction in beta-diversity occurs along first 6 months of follow-up

NMDS ordination plots show degree of similarity among samples from the baseline dataset (48 PHI<6, 14 PHI>6, 71 CHI_noART, 27 CHI_ART and 54 NEG) using the Bray-Curtis, Weighted UniFrac and UnWeighted UniFrac distances.



Boxplots (median±IQR) show values for three distance metrics: bray-curtis, euclidean and weighted UniFrac. Each dot represents a pairwise distance between two samples within each group. Only PHI samples from baseline dataset are considered. Samples are categorised based on their month of collection. Distances are calculated on relative abundance of bacterial genera and its phylogenetic tree for the Weighted UniFrac distance.

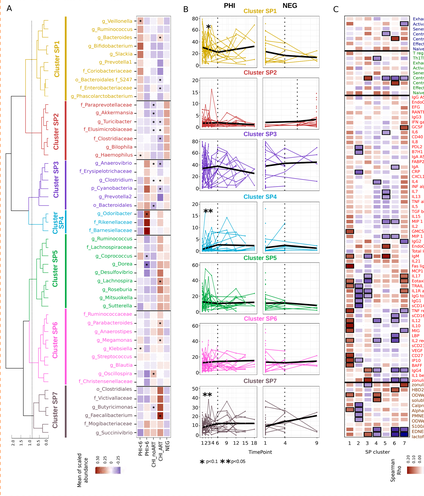


3) Increase of anti-inflammatory bacteria abundance along with a reduction of pro-inflammatory bacteria after hyper-acute dysbiosis following HIV-1 infection

A. Color gradient represents mean value of scaled individual relative abundance values (forced to mean=0 and sd=1) per bacterial genera and group. Bacterial abundance vectors across the five groups were further used to compute Euclidean distances among bacteria. Distance matrix was used to cluster bacteria using Ward.D2 algorithm and resulting dendrogram was split into 7 clusters (SP1 to SP7) using a phylogenetic distance value of 0.8. Comparisons of mean values were made for each group against the HIV-1 negative group (asterisks on colored boxes, p<0.05 on Wilcoxon Rank Sum Test)

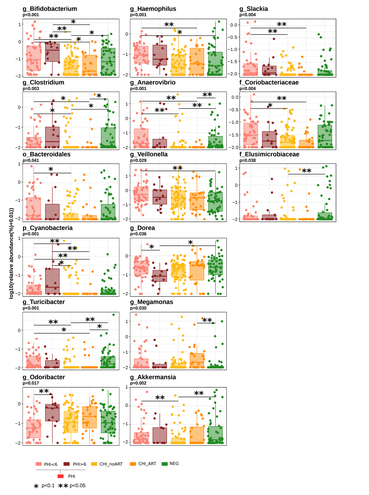
B. Results of LMM assessing longitudinal behavior of clusters' abundances in the NEG (n=67) and PHI (n=149) groups.

C. Spearman's correlation between bacterial clusters and immune measurements (T $CD8^+$ cell subset in blue, T $CD4^+$ cell subset in green, measurements in blood in red and measurements in feces in brown).



4) Bacterial genera signature on HIV-1 infected groups

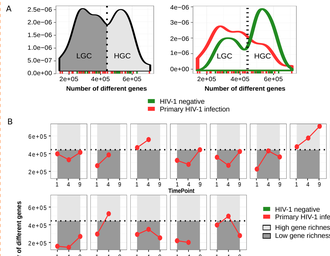
Boxplots (median±IQR) represent relative abundance of bacterial genera at baseline. Bacterial genera named "unclassified" are identified by their closest taxonomic level identification. Only bacterial genera with a Kruskal-Wallis p-value <=0.05 are shown.



Whole metagenome sequencing

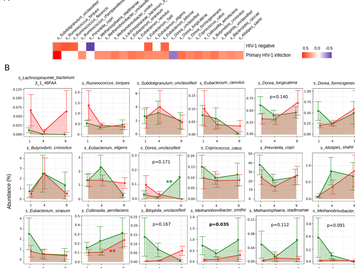
1) Reduced gene richness in primary HIV-1 infected subjects and delayed recovery compared to HIV-1 negative subjects along first 9 months of follow-up

A. Density plot on the left shows distribution of samples (n=54, 35 PHI and 19 NEG) based on their observed gene richness value (i.e. number of different genes detected using shotgun sequencing data). Colored individual bars below the plot indicate at which group does each sample belongs to. Density plot on the right shows same distribution but split by group. Dotted black line set at 444219 on the x axis defines the minimum local peak of density. Samples with gene richness above this threshold are categorized into high gene count (HGC), and samples with gene richness below are categorized as low gene count (LGC). **B.** Each box corresponds to an individual and each dot to a sample. Connected dots along the x axis represent variation in observed gene richness value per subject along follow-up. Dark gray colored area represents LGC zone whereas light gray colored area represents HGC zone.



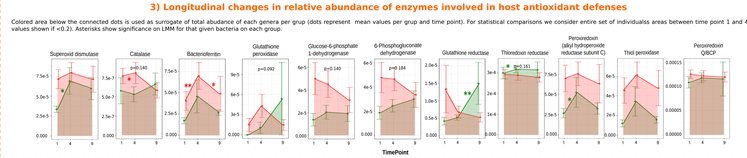
2) Bacterial genera positively correlated to gene richness are depleted in PHI compared to NEG individuals.

A. Color code represents Spearman's correlation Rho value between the bacterial taxa and observed gene richness. To test correlation all samples available were considered: 35 in the PHI group and 19 in the NEG group. **B.** Colored area below the connected dots is used as surrogate of total abundance of each genera per grup (dots represent mean values per group and time point). For statistical comparisons we consider entire set of individuals areas between time point 1 and 4 (p-values shown if <0.2). Asterisks show significance on LMM for that given bacteria on each group.



3) Longitudinal changes in relative abundance of enzymes involved in host antioxidant defenses

Colored area below the connected dots is used as surrogate of total abundance of each genera per grup (dots represent mean values per grup and time point). For statistical comparisons we consider entire set of individuals areas between time point 1 and 4 (p-values shown if <0.2). Asterisks show significance on LMM for that given bacteria on each group.



qPCR on eukaryotic viruses

Adenovirus dominated viral expansion following primary HIV-1 infection

Numbers (percent) of subjects with detectable virus in feces by qualitative commercial real-time PCR. To avoid ascertainment bias due to diferent follow-up between groups, only the frst fecal sample available for testing is used for comparisons. Fisher's pairwise comparisons are relative to HIV-1 negative.

	Primary HIV-1 infection	Chronic HIV-1 infection		HIV-Negative
		ART-naive	On ART	
Adenovirus	26/49 (53.2%)**	36/71 (50.7%)**	12/27(44.4%)**	11/55 (20.0 %)
Cytomegalovirus	3/49 (6.1%)	8/71 (11.3%)*	2/27 (7.4%)	1/55 (1.8%)
Enterovirus	1/43 (2.4%)	4/19 (21.1%)*	1/25 (4.0%)	2/45 (4.4%)
Human Herpes Virus 6A, 6B & 8	8/49 (0%)	0/71 (0%)	0/71 (0%)	0/55 (0%)

★ p<0.1 ★★p<0.05

CONCLUSION

Primary HIV-1 infection is associated with virome expansion, transient loss of bacterial taxonomic richness and long-term reductions in microbial gene richness, all of which occur during hyperacute phases of HIV-1 infection.

Muntsa Rocafort [1,2], Marc Noguera-Julian [1,2,3], Javier Rivera [1,3], Lucía Pastor Palomo [1,2,4,5,6], Yolanda Guillén [1], Jost Langhorst [7], Mariona Parera [1], Inacio Mandomando [4], Jorge Carrillo [1,4], Victor Urrea [1], Cristina Rodríguez [1], Maria Casadellà [1], María Luz Calle [3], Bonaventura Clotet [1,2,3,6], Julià Blanco [1,3,6], Denise Naniche [4,5], Roger Paredes [1,2,3,6★]

[1]irsiCaixa AIDS Research Institute, Ctra de Canyet s/n,Badalona, Catalonia, Spain; [2]Universitat Autònoma de Barcelona, Bellaterra, Catalonia, Spain; [3]Universitat de Vic-Universitat Central de Catalunya, Vic, Catalonia, Spain; [4]Barcelona Institute for Global Health (ISGLOBAL), Barcelona, Catalonia, Spain; [5]Institut Germans Trias i Pujol (IGTP), Hospital Germans Trias i Pujol, Badalona, Catalonia, Spain; [6]Centro de Investigação em Saúde da Manhiça (CISM), Maputo, Mozambique; [7]University of Duisburg-Essen, Kliniken Essen-Mitte, Internal Medicine V, Am Deimelsberg, Essen, Germany; [8]Infectious Diseases Unit, Hospital Universitari Germans Trias i Pujol, Ctra de Canyet s/n, Badalona, Catalonia, Spain