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***Basic Study***

**Effects of viremia and CD4 recovery on gut “microbiome-immunity” axis in treatment-naïve HIV-1-infected patients undergoing antiretroviral therapy**

Russo E *et al*. Gut“microbiome-immunity”axisin HIV-1 infectedpatients

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**Abstract**

BACKGROUND

Human immunodeficiency virus type 1 (HIV-1)infectionischaracterizedbypersistentsystemicinflammationandimmuneactivation,eveninpatientsreceivingeffectiveantiretroviraltherapy(ART).Convergingdatafrommanycross-sectionalstudiessuggestthat gutmicrobiota(GM)changescanoccurthroughoutincludinghumanimmunodeficiencyvirus(HIV)infection,treatedbyART;however,theresultsarecontrasting.Forthefirsttime,wecomparedthefecalmicrobialcomposition,serum andfecalmicrobialmetabolites,andserumcytokineprofileof treatment*-*naïvepatientsbeforestartingARTandafterreachingvirologicalsuppression,after24wkofARTtherapy.Inaddition,wecomparedthemicrobiotacomposition,microbialmetabolites,andcytokine profileofpatientswithCD4/CD8ratio< 1(immunologicalnon-responders [INRs])andCD4/CD8> 1(immunologicalresponders [IRs]),after24wkofARTtherapy.

AIM

Tocompareforthefirsttimethefecalmicrobialcomposition,serumandfecalmicrobialmetabolites,andserumcytokineprofileoftreatment*-*naïvepatientsbeforestartingARTandafterreachingvirologicalsuppression(HIVRNA<50copies/mL)after24wkofART.

METHODS

Weenrolled12treatment*-*naïve HIV-infectedpatientsreceivingART(mainlybasedonintegraseinhibitors).Fecalmicrobiotacompositionwasassessedthroughnextgenerationsequencing.Inaddition,acomprehensiveanalysisofabloodbroad-spectrumcytokine panelwasperformedthroughamultiplexapproach.Atthesametime,serumfreefattyacid(FFA)andfecalshortchainfattyacid levelswereobtainedthroughgas chromatography-mass spectrometry.

RESULTS

Wefirstcomparedmicrobiotasignatures,FFAlevels,andcytokineprofilebeforestartingARTandafterreachingvirologicalsuppression.Modestalterationswereobservedinmicrobiotacomposition,inparticularin theviralsuppressioncondition,wedetectedanincreaseof *Ruminococcus* and *Succinivibrio* andadecreaseof *Intestinibacter*.Moreover,inthesamecondition,wealsoobservedaugmentedlevelsofserumpropionicandbutyricacids.Contemporarily,areductionofserumIP-10andanincreaseofIL-8levelsweredetected in theviralsuppressioncondition.In addition,thesamecomponentswerecomparedbetweenIRsandINRs.Concerningthemicroflorapopulation,wedetectedareductionof *Faecalibacterium* andanincreaseof *Alistipes* inINRs.Simultaneously,fecalisobutyric,isovaleric,and2-methylbutyricacidswerealsoincreasedinINRs.

CONCLUSION

OurresultsprovidedanadditionalperspectiveabouttheimpactofHIVinfection,ART,andimmunerecoveryonthe“microbiome-immunityaxis”atthemetabolismlevel.Thesefactorscanactasindicatorsoftheactiveprocessesoccurringinthegastrointestinaltract.IndividualswithHIV-1infection,beforeARTandafterreachingvirologicalsuppressionwith24wkofART,displayedamicrobiotawithunchangedoverallbacterialdiversity;moreover,theirsystemicinflammatorystatusseems nottobe completelyrestored.Inaddition,weconfirmedtheroleoftheGMmetabolitesinimmunereconstitution.

**KeyWords:**HIV;Antiretroviraltherapy;Microbiome-immunityaxis;Microbiota;Cytokines;Shortchainfattyacid;Inflammation;Immunologicalresponders;Viremia

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**CoreTip:**Eveninpatientsreceivingeffectiveantiretroviraltherapy(ART),human immunodeficiency virus type 1infectionischaracterizedbypersistentsystemicinflammationandimmuneactivation.ChangesinthegutmicrobiotacanoccurwithincludinghumanimmunodeficiencyvirusinfectionandtreatmentwithART;however,thedataarestillconflicting.Forthesereasons,wecomparedthefecalmicrobialcompositionandserumcytokine profileoftreatment*-*naïvepatientsbeforestartingARTandaftervirologicalsuppression.Finally,weevaluatedthemicrobiotacomposition,microbialmetabolites,andcytokine profileofpatientswithCD4/CD8ratio< 1andCD4/CD8> 1(immunologicalresponders).

**INTRODUCTION**

Themutualinteractionbetweenthehumanmicrobiotaandtheimmunesystemdefinestheso*-*called “microbiome-immuneaxis”.Thisaxishasalsobeenassociatedwithseveraldiseases,includinghumanimmunodeficiencyvirus(HIV)infection[1].Indeed,akeyplaceforHIVreplicationisthegastrointestinaltract. HIVreplicationinthegastrointestinaltractresultsinaseveredepletionofCD4+Tcellsthatleadstodecreasedfunctionoftheepithelialbarrier,allowingmicrobesandmicrobialproductstobetranslocated,whichcontributestothechronicinflammatoryresponse[2].HIVreplicationcanalsoresultinamicrobialdysbiosiscondition[3-5],whichhasbeencorrelatedwithincreasesinmarkersofdiseaseprogression,immuneactivation,andmicrobialtranslocation[3,5-7].Notably,HIV-infectedpeopleharbouradistinctgutmicrobiota (GM)[8,9]witha *Prevotella-*richcommunitycomposition,typicallyobservedinindividualsfromagrarianculturesorwithcarbohydrate-rich,protein-andfat-poordiets[10].Inaddition,thesignificantsubversionofthe *Bacteroidetes* and *Proteobacteria* phyla,withanimbalanced *Prevotella/Bacteroides* speciesratioandanabundancein *Enterobacteriaceae,* isoneofthemostpersistentchangesdocumentedinuntreatedHIVinfection[11-13].Moreover,theincreasednumberofgut-residentbacteriacapableofdirectlyproducinginflammationcanbeaprobablemechanisticlinkbetweenHIV-associateddysbiosisandhighsystemicimmuneactivation[14].However,convergingdatafromcross-sectionalstudiessuggestthattheGMcompositionanditsrelatedimmuneresponsecanchangeovertheprogressionofHIVinfection.Inparticular,correlatingthecompositionofthegastrointestinaltractmicrobiometoimmuneactivation,circulatingbacterialproductsandclinicalparameters,adecreaseofcommensalspecies,andagainofpathogenictaxawasobservedinHIV+subjectscomparedtocontrols[15].Additionally,analysingthefunctionalgenecontentof theGMinHIV+patientsandthemetabolicpathwaysofthebacterialcommunityassociatedwithimmunedysfunction,themetagenomesequencingrevealedanalteredfunctionalprofilewithsignificantinteractionsbetweenthebacterialcommunity,theiralteredmetabolicpathways,andsystemicmarkersofimmunedysfunction[16].Furthermore,analysingtheassociationsbetweenthe innatelymphoidcell (ILC)cytokinesandmeasuresofvirologic,immunologic,andmicrobiomeindices,itwasobservedthatinflammatoryILCscontributetogutmucosalinflammationandepithelialbarrierbreakdown,importantfeaturesofHIV-1mucosalpathogenesis[17].DespitegrowingevidencethattheGMhasaroleinHIVpathogenesis[11,18-20],theresults werecontrasting,withsomestudiessuggestinganinfluenceandothersnoHIVinfluenceonmicrobialdiversity[1,21]andcomposition[22,23].However,manystudiesontheGMinHIV-infectedpatientsareoftencarriedoutwith alackofadjustmentforconfoundingfactors,suchasdietanduseofdrugs[24,25].

Currently,antiretroviraltherapy(ART)hasincreasedthelifeexpectancyofHIV-infectedpatients,approximatingittothatofthegeneralpopulation[26].Interestingly,chronicinflammationandGMalterationspersistinpatientsvirologicallysuppressedbyART[27].Thesedataimplicatethatre-shapingthemicrobiotamaybeanadjuvanttherapyinpatientscommencingsuccessfulART[28].Ontheotherhand,suppressiveARTappearstohavealimitedeffectontherestorationof theGM[13,25,29,30].AlthoughthegutmicrobialcompositionofART-treatedpeoplediffersfromthatofuntreatedpeople,theformeralsohaveadifferentmicrobialcommunitystructure compared totheHIV-uninfectedpopulation[31,32].ThesefindingsraisethepossibilitythatpersistentgutdysbiosismayplayaroleinthedevelopmentofresidualclinicalillnessafterART.

Currently,theCD4/CD8ratioisconsidered oneofthebest-usedmarkersofimmunereconstitution.Notably,alowCD4/CD8ratioisassociatedwithanincreasedriskofnon-AIDS-relateddiseases[33].Furthermore,thedifferencesbetweentheelementsofthemicrobiome-immuneaxisbetweenpatients withnormalizedornon*-*normalized CD4/CD8ratioduringARThavenotbeenelucidatedsofar[34,35];however,thisquestionisrecognizedasacurrentresearchgap.

Moreover,withabetterunderstandingofthemicrobiota-immuneaxis,itisnowknownthatinadditiontotheintestinalfloraitself,itsmetabolitesarealsoinvolvedinregulatingvitalhostactivities,suchasenergymetabolism,cell-to-cellcommunication,andhostimmunity.Short-chainfattyacids(SCFAs)areimportantmetabolitesabletomodulatetheproductionofimmunemediators,suchaskeycytokinesfortherepairandmaintenanceofepitheliumintegrity[36].Inaddition,theSCFAsmodulatetheactivityofTcellsanddecreasetheoverexpressionofhistonedeacetylase,particularlybutyricandvalericacids[37].SCFAsareanimportantlinkbetweenmicrofloraandtheimmunesystem;theyinvolvedifferentmolecularmechanismsandcellulartargets,areessentialforthemaintenanceofintestinalhomeostasis,andfinallyplayaroleinHIVinfection[38].

Thepurposeofthisprospectiveobservationalstudywastocompareforthefirsttimethefecalmicrobialcomposition,serumandfecalmicrobialmetabolites,andserumcytokineprofileoftreatment*-*naïvepatientsbeforestartingARTandafterreachingvirologicalsuppression(HIVRNA<50copies/mL)after24wkofART.AnadditionalaimwastocorrelatetheGMcomposition,microbialmetabolites,andcytokineprofileofpatientswithCD4/CD8ratio<1andCD4/CD8>1afterantiretroviraltherapy.

**MATERIALSANDMETHODS**

***Patients***

Thestudypopulation,composedof12treatment*-*naïve HIV-infectedpatients receivingARTmainlybasedonintegraseinhibitors,wasenrolledbetweenApril2018andMay2019attheDepartmentofInfectiveandTropicalDiseaseatUniversityHospitalofCareggi,Florence,Italy(Table1).Thestudywasapprovedbylocalinstitutionalreviewboardsandwritteninformedconsentwasobtainedfrompatientsbeforeparticipation(RifCEAVC15035).

Weconductedaprospectiveobservationalcohortstudycomparingthechangesoccurringinthefecalmicrobiota,serumandfecalSCFA,serumfreefattyacids(FFAs),andserumcytokinesofpatientswithHIV-1infectionbeforeART(T0)andafter24wk(T1).Inaddition,patientsweredividedintotwogroups according towhethertheywereimmunologicalresponders(IRs, *n* =6)ornot(INRs, *n* =6)(INRsandIRs,basedonthenormalizationofCD4/CD8ratio:<1or≥1after24wkofART,respectively).Patientswhohadusedantibiotics,probiotics,orprebioticsorhadexperienceddiarrhoeaordigestivesymptomswithintheprevious1mo wereexcluded.

Personaldata,ARTregimen,HIV-RNAvalues,andnumberofCD4+andCD8+TcellspriortoARTstarting andatthetimeofvirologicsuppression wereincludedintheanalysis(Table1).Inthispilotexploratorystudy,noformalsamplesizecalculationwasperformed.AllpatientsfollowedaMediterraneandiet.

Plasma HIV-RNA wasmeasured usingTestv1.5RocheCOBASAmpliPrep,RocheTaqManHIV-1Testv2.0(RocheDiagnostics,Branchburg,NJ,UnitedStates)andSiemensVersantKPCR(SiemensHealthcareGmbH,Erlangen,Germany),withlowerlimitsofdetectionof50,20,and37copies/mL,respectively.

TheTcellcountsofpatientsweredeterminedusingaFACScantoflowcytometer(BDImmunocytometrySystems)[10].Immunophenotypingofperipheralbloodlymphocyteswasanalysedbythree-colorflowcytometry(EpicsXLFlowCytometry System;BeckmanCoulter,UnitedStates)aspreviouslydescribed[39].FreshlycollectedEDTAanticoagulatedwholebloodwasincubatedandtestedwithapanelofmonoclonalantibodiesdirectedagainstfluoresceinisothiocyanate/phycoerythrin/peridininchlorophyllproteincombinationsofCD3/CD4/CD8,CD3/CD16CD56/CD19,HLA-DR/CD8/CD38,andCD4/CD8/CD28andisotypecontrols(Immunotech,France).

Ateachtimepoint(0 and24wkafterstudyenrolment),wecollectedbloodandfecalsamples.Aftercollection,stoolsampleswereimmediatelyfrozenandstoredat−80 °CuntilDNAextraction.FecalsampleswereusedtoassessthemicrobiotacompositionandSCFAs, andwhilebloodsampleswereusedtomeasureSCFAsandFFAsandapanelof27selectedcytokines.

***Study follow-up***

Patientsunderwent medicalvisitsat0 and24 wk afterstudyenrolment.They alsounderwentacomprehensivephysicalexaminationandmedicalhistory inquiry,urinetoxicologypanel testing,clinicallaboratorytestsincludingplasmaHIVRNA,specimencollection,anddetailedbehaviouralquestionnaire survey.Demographicandclinicaldatawerecollectedinaspecificquestionnaireandreportedinanappropriatedatabase, includingthetimepointoffollow-upinmonths;theparticipant’sgender,age,weight,andheight;CD4+andCD8+Tcellcounts;theCD4/CD8ratio;HIV-1RNAlevels,art,andantibioticuse.Ifsubjectshadtostartantibiotics,theyprovidedalastfecalsampleandthestudyfollow-upwasimmediatelyterminated

***Fecal microbiota characterization***

TotalgenomicDNAwasextractedfromfrozen(-80 °C)stoolsamples,collectedatdifferenttimepoints(weeks0 and24; T0 and T24),usingtheDNeasyPowerLyzerPowerSoilKit(Qiagen,Hilden,Germany)accordingtothemanufacturer’sinstructions.ThequalityandquantityofpurifiedDNAwereassessedusingtheNanoDropND-1000(ThermoFisherScientific,WalthAP,US)andtheQubitFluorometer(ThermoFisherScientific),respectively.

ExtractedDNAsamplesweresenttoIGATechnologyServices(Udine,Italy)whereampliconsofthevariableV3–V4regionofthebacterial16SrRNAgeneweresequenced(2×300bppaired-end)ontheIlluminaMiSeqplatform,accordingtotheIllumina16SMetagenomicSequencingLibraryPreparationprotocol[40].

SequencingresultswereanalysedusingtheQIIME2suite(QuantitativeInsightsIntoMicrobialEcology)[41].Briefly,followingrawreadsdenoising(*i.e.*,estimationoferrorrates,removalofchimericandsingletonsequences, andjoinofdenoisedpaired-endreads)usingDADA2(DivisiveAmpliconDenoisingAlgorithm2)[42],denoisedreadsweredereplicatedandampliconsequencevariants(ASVs)wereinferred.TaxonomicclassificationofinferredASVswasperformedusingaNaiveBayesclassifiertrainedontheSILVA16Sreferencedatabase(release132)(https://www.arb-silva.de/documentation/release-132/).

***Evaluation of fecal short chain fatty acids and serum free fatty acids by gas chromatography-mass spectrometry***

Thefecal SCFAs,inparticularacetic,propionic,butyric,isobutyric,isovaleric,2-methylbutyric,valeric,andhexanoicacids,wereanalyzed using anAgilentGC-MSsystemcomposedwith a5971singlequadrupolemassspectrometer, a5890gas-chromatograph,and a7673autosampler.Thechemicals,GC-MSconditions,andcalibrationsparametersarereportedinsupportinginformation(Tables S1-S4)[43].Fecalsampleswerecollectedin15*-*mLFalcontubesandstoredat-80°C.Justbeforetheanalysis,eachsamplewasthawed,weighted(between0.5-1.0g),andaddedtosodiumbicarbonate10mmol/Lsolution(1:1w/v)ina1.5mLcentrifugetube.Theobtainedsuspensionwasbrieflystirredinavortexapparatus,extractedinanultrasonicbath(for5min),andthencentrifugedat5000rpm(for10min).Thesupernatantwascollectedandtransferredinto a1.5mLcentrifugetube(samplesolution).TheSCFAswerefinallyextractedasfollows:Analiquotof100µLofsamplesolutionwasaddedto50μLofinternal standardmixture,1mLoftert-butylmethylether,and50µLof1.0mol/LHClsolutionin a1.5mLcentrifugetube.Afterwards,eachtubewasshakeninavortexapparatusfor2min andcentrifugedat10000rpmfor5min,andfinallythesolventlayerwastransferredinto anautosamplervialandanalyzedby theGC-MSmethod.Eachsamplewaspreparedandprocessed,bythemethoddescribedabove,threetimes.Inaddition,serum FFAs,classifiedasSCFAs(acetic,propionic,butyric,isobutyricisovaleric,2-methylbutyri,andvalericacids),mediumchainfattyacids(MCFAs;hexanoic,heptanoic,octanoic,nonanoic,decanoic,anddodecanoicacids),andlongchainfattyacids(LCFAs;tetradecanoic,hexadecanoic,andoctadecanoicacids)wereanalyzedwithourpreviousdescribedGC-MSprotocol[44].Thechemicals,GC-MSconditions,GC-MSmethod,andcalibrationsparametersarereportedinsupportinginformation(TablesS5-S7).

Justbeforetheanalysis,eachsamplewasthawed.TheFFAswereextractedasfollows:Analiquotof300µLofplasmasamplewasaddedto10μLofinternal standard mixture,100μLoftert-butylmethylether,and20µLof6MHClplus0.5mol/LNaClsolutionin a0.5mLcentrifugetube.Afterwards,eachtubewasstirredinvortexfor2min andcentrifugedat10000rpmfor5min,andfinallythesolventlayerwastransferredinto avialwith amicrovolumeinsertandanalyzed.

***Molecular inflammatory response in serum***

Theinflammatoryresponse inserumsamplesofpatientsandhealthycontrolswas evaluated using aspecificallyassembledkitProCartaPlexMixMatchHuman27PanelforLuminexMAGPIXdetectionsystem(Affymetrix,eBioscience) followingthemanufacturers'instructions.

Indetail,thepanelincludedmacrophageinflammatoryprotein-1α(MIP-1α),interleukin(IL)-27,IL-1β,IL-2,IL-4,IL-5,interferongamma-inducedprotein10(IP-10),IL-6,IL-8,IL-10,IL-12p70,IL-13,IL-17A,interferon(IFN)-γ,IFN-α,tumornecrosisfactor-α(TNF-α),granulocyte-macrophagecolonystimulatingfactor(GM-CSF),monocytechemotacticprotein1(MCP-1),IL-9,P-selectin,IL-1α,IL-23,IL-18,IL-21,solubleintercellularadhesionmolecule-1(sICAM-1),IL-22,andE-selectin.

Allmeasurementswereperformedinablindedmannerbyalaboratorytechnicianwhowasexperiencedinexecutingthetechnique.Thelevelsofcytokineswereestimatedusinga5-parameterpolynomialcurve(ProcartaPlexAnalyst1.0)**.**Avalueunderthelowlimitofquantification(LLOQ)wasconsideredas0pg/mL.

***Statistical analysis***

StatisticalanalysesonASVsrepresentingthebacterialcommunitywereperformedinR(RCoreTeam,2014)withthehelpofthepackagesphyloseq1.26.1[45] andDESeq21.22.2[46],andotherpackagessatisfyingtheirdependencies,inparticularvegan2.5-5[47].RarefactionanalysisonASVswasperformedusingthefunctionrarecurve(step50reads), andfurtherprocessedtohighlightsaturatedsamples(arbitrarilydefinedassaturatedsampleswithafinalslopeintherarefactioncurvewithanincrementinASVnumberperreads<1e-5).Fortheclusteranalysis(completeclusteringoneuclideandistance)oftheentirecommunity,theOTUtablewasfirstnormalizedusingthetotalASVcountsofeachsampleandthenadjustedusingsquareroottransformation.ThecoveragewascalculatedbyGood'sestimatorusingtheformula (1- *n*/*N*)×100,where *n* isthenumberofsequencesfoundonceinasample(singletons),and *N* isthetotalnumberofsequencesinthatsample.

Richness,Shannon,Chao1,andevennessindiceswereusedtoestimatebacterialdiversityineachsampleusingthefunctionestimate\_richnessfromphyloseq[45].TheevennessindexwascalculatedusingtheformulaE=S/Log(R),whereSistheShannondiversityindexandRisthenumberofASVsinthesample.DifferencesinallindicesweretestedusingapairedWilcoxonsigned-ranktest.ThedifferentialanalysisofabundanceattheASVsaswellasatthedifferenttaxonomicranks(createdusingthetax\_glomfunctioninphyloseq)wasperformedwithDESeq2[46]usingatwogroupblockedbypatientdesigninordertoperformapairedtest[48].

Inaddition,thesoftwareGraphPadPrism(v.5)andStatgraphicsCenturionXVIsoftwarewereusedforimmunological dataanalysis.Numericaldataarepresentedas themean±SD.Theconcentrationsofseveralcytokinesinsomeofthesampleslaybelowthecurvefitofthestandards.Toavoidthebiasthatwouldhavebeenintroducedbyexcludingthesedata,theconcentrationsoftheimplicatedcytokineweresetathalfofthelowercutoffofthetestsystem,whichwasusuallyabout1pg/mL.Outliersattheotherendofthespectrum(higherthanthemean±SD)wereidentified *via* boxplotsandwereexcludedfromthestatisticalanalysis.ThecomparisonsbetweendependentgroupswereevaluatedbytheWilcoxonmatchedpairstest,whilethecomparisonsbetweentheindependentgroupswereassessedbytheMann-Whitneytest. A *P* valuelessthan0.05wereconsideredstatisticallysignificant.

***Data availability statement***

The16SrRNAsequencedatasethasbeendepositedintheNCBISequenceReadArchive(SRA)databaseandisavailableundertheBioProjectaccessionnumberPRJNA731648.

**RESULTS**

***Comparison of fecal microbiota and metabolic and inflammatory profiles after ART***

**ModestdifferencesinspecificfecalmicrobiotataxaassociatedwithHIVviremia:**Inthefirstpartofourstudy,wecomparedthefecalmicrobiota andmetabolicandinflammatoryprofilebeforeandafterARTstarting,inordertoexaminepotentialchangesresultingfromHIVinfectionand ARTtherapy.WefirstanalysedthelongitudinalvariationoffecalmicrobiotapopulationinthesamepatientsatT0(HIV+viremia- RNA>50copies/mL),definedas“highviremia”condition,andT24(HIV+suppression-RNA≤50copies/mL),definedas“viralsuppression”condition.ThealphadiversityofsamplesdidnotdisplaysignificantdifferencesforChao,Shannon, andevenness indices(Figure1).Theanalysisofthetaxonomiccompositionrevealedthatmorethan99%ofthesequencescollectedwereclassifiedintofourphyla: *Firmicutes* (65.46%), *Bacteroidetes* (21.54%), *Actinobacteria* (9.40%), and *Proteobacteria* (2.72%).Inordertoinvestigatesimilarityofpatients’microbiotaabundanceprofilesandtostudythepairednatureofsampling(*i.e.*,highviremiacondition *vs* viralsuppressioncondition),aclusteranalysisand PCoAonnormalizedASVcountswereperformed.

Thehierarchicalclusteringevidencedthatmicrobiotawasnotsufficientlyalteredaftertreatment(24wk)tobreakindividualcompositionsapart,resultinginaperfectmatchingofthetwotimepointsfromthesamepatient(Figure2A).ThisresultwasalsoconfirmedbythePCoA(Figure2B), whichshowedasubstantialproximityofeachpatientatT0andT24,indicatingthat,overall,theabundanceprofileofthesinglepatientwasnotaffectedbythe24*-*wktherapy.

Ontheotherhand,thepairedcomparisonoftheabundanceofsinglemicrobialranksrevealedsomesignificant(adj. *p* <0.05,abs(logFC)≥1)differencesbetweenthetwosamplesgroups.Inparticular,thegenera *Ruminococcus 2* and *Succinivibrio* werefoundtobesignificantlyincreasedinhigherviralsuppressioncondition.Onthecontrary,viralsuppressionwasrelatedwithadecreaseinthe *Intestinibacter* genus(medianabundance,~1%)(Figure3).

**AnalysisoffecalSCFAs displaysnodifferentlayoutbetween“highviremia”and“viralsuppression”conditions:**Aswenoticedminorchangesinfecalmicrobiomeprofile(justattheorderandgenuslevels),wewonderediftheGMmetabolicactivityhad beenalteredaswell,andwhetherthisactivitymightbemaskedbysimplyexaminingthemicrobiotacomposition.InordertoevaluatethepresenceofalterationsinGMmetabolicactivity,thelevelsofmicrobiallinearandbranchedSCFAsweremeasured infecalsamplesforeachpatient.However,theanalysisof linearSCFA(acetic,propionic,butyric, andvalericacids),andbranchedSCFA(isobutyric,isovaleric, and2*-*metilbutyricacids)abundancedidnotrevealanysignificantchangeafter24wkoftherapyforeachpatient.

**AnalysisofserumFFAs revealsasignificantlydifferentsubgroupofSCFAsbetween“highviremia”and“viralsuppression”conditions:**AswedidnotreportalterationsinthecompositionoffecalSCFAs,wewantedtoobserveiftherewereanyotheralterationsinmetabolicoutput,byanalyzingbothmicrobialandhostderivedFFAsinserum.Asknown,theimpairmentofgutintegrityduetodysbiosiscondition,leadstotranslocationofmicrobialelementsfromtheintestinalmucosatothebloodstream,whichisconsideredamajordrivingforceofchronicimmuneactivation[49]eveninpatientssuccessfullytreatedwithARTandachievingstablevirologicalsuppression[2].

TheanalysisofserumFFAlevelsshowedasignificantchangeoftwoSCFAsatT24comparedtothebaseline.Inparticular,propionicandbutyricacidswereincreasedinviralsuppressioncondition(Figure4).

**Inflammatoryprofilebetweenhighviremiaandviralsuppressionconditions:**Asknown,gutmicrobialdysbiosisislinkedtoaberrantimmuneresponses,asalterationsintheGMmayinducetheinterruptionofgutepithelialbarrierintegritywithsubsequentmicrobialtranslocation,increasedinflammation,andimmuneactivation,whichareoftenaccompaniedbyabnormaldifferentiationofimmunologicalcells[6,50].Sincewedetectedsignificantvariationsofmicrobialcommunitiesbetweenhighviremiaandviralsuppressionconditions,wedecidedtocharacterizealsotheserumimmunologicalprofile byevaluatingapanelof27cytokinesbetweenthetwomentionedconditions.Amongthe27cytokinesexamined,wedetectedasignificantreductionofIP-10(*p* =0.0244)andasignificantincrementofIL-8levels(*P* =0.0547)in thehighviremiasetting(Figure5).

***Association of GM composition and metabolic and inflammatory profiles with CD4+ T-cell counts***

**CorrelationbetweenfecalmicrobiotaandCD4/CD8ratio:**Inthesecondpartofourstudy,wedividedourcohortofpatientsintotwogroups:Immunologicalresponders(IRs)andimmunologicalnon-responders(INRs),basedon the CD4/CD8ratio>1or<1.Inthiscondition,theanalysisofmicrobiotarevealedthat,consideringonlytaxawithanoverallabundancehigherthan1%,membersofthe *Faecalibacteria* genusweresignificantlyreduced(adj. *p* <0.05,logFC=1.32)whilemembersofthe *Alistipes* genusweresignificantlyincreasedinresponders(adj. *p* <0.05,logFC=2.5).

**DifferentbranchedSCFAprofiles inserumandfecalsamples betweenIRsandINRs:**AsweobservedsignificantvariationsinthecompositionofthefecalmicrobiotabetweenIRsandINRs,weassessediftherewereanyotheralterations inthefecalandserummicrobialmetabolitesaslinearandbranchedSCFAsderivedfrombacterialmetabolism.Wedocumentedsignificantchangesinisobutyric(*p =* 0.01),isovaleric(*p =* 0.04),and2-methylbutyric(*p =* 0.04)acids,whichwereincreasedinIRfecalsampleswhile wedidnotdetectsignificantdifferencesinserumsamples(Figure7).

**Inflammatoryprofileshowsnosignificantdifferences betweenIRsandINRs:**SincewedetectedsignificantvariationsofmicrobialcommunitiesbetweenIRsandINRs,wealsoevaluatedtheserumimmunologicalprofile.However,cytokinelevelsdidnotshowsignificantvariationsbetweenthe IRsandINRs.

**DISCUSSION**

Currently,themechanismsregulatingtheinterplaybetweenthehostimmunesystemandHIV-1,aswellastheexactchangesoccurringintheGMcompositionandfunctionality,remaintobedefined.Toclarifytheintricaterelationshipsbetweentheactorsofthe“microbiota-immunity”axis,weexaminedmicrobiotacompositionandfunctionality(SCFAs),seruminflammatoryresponse,andFFAcompositioninindividualsundergoing ARTindifferentHIVinfectionsettings.

Today,manystudiesonmicrobiotahavebeenperformedchieflycomparingHIV-infectedanduninfectedindividuals,revealingareducedGMdiversity(theso-calledHIV-associateddysbiosis)andanindependentassociationbetweenalpha-diversityofmicrobiotaandperipherallevelsofCD4+Tcellcountintreatment*-*naïveHIV*-*infectedpatients[28].However,cross-sectionalstudiesmaynotbesuitabletoprovideinformationaboutcause-and-effectrelationships,whereaslongitudinalonescouldbemorevalidforexaminingsuchrelationships.Besides,thereisalackofhumanlongitudinalobservationsofthe“microbiota-immunity”axisbeforeandafterfirstARTadministration.Onlyinfewlongitudinalstudies,whereHIV-1-infectedparticipantswerefollowedafterARTstarting,dataobtainedonbacterialflorashowedthatshiftsinthefecalmicrobiotapersistedinanumberofpatients[10,28].Ontheotherhand,arecentstudybyDillon *et al*[14]failedtofindasignificantchangeinasingletimepointstudyofthestoolofHIV-1-infectedpatients.

Inthisstudy,wefirstperformedalongitudinalinvestigationevaluatingtheGMbeforethetreatmentandafter“viralsuppression”(T24).AccordingtothelongitudinalstudyconductedbyDillon *et al*[14],ourresultsshowedmodestchangesintheGMcompositionafterART;indeed,wedidnotassesssignificantdifferencesinphylumcomposition.However,thepairedcomparisonoftheabundanceofsinglebacterialtaxarevealed asignificantalterationatthegenuslevelbetweenthetwosamplegroups(Figure3).Inparticular,thegeneraof *Ruminococcus,* and *Succinivibrio* weresignificantlyincreasedafterARTandtheviralsuppression.Conversely,thegenusof *Intestinibacter* was significantlydecreasedinthesamecondition.Wehypothesizethattheslightchangebetweenthetwogroupsmaybeduetopersistentinflammation(relatedtomicrobialtranslocationandreducedimmunoregulatoryfunction),HIVlatencythroughoutthegut,anddirecteffectsofantiretroviraldrugsonthebacterialpopulation.Moreover,ourresultsareinaccordancewithotherlongitudinalpreviousstudiesinnon-humanprimates,whichallowedtocontrolforconfoundersaffectinghumanstudies[51,52].Wealsoreportedanincreaseofthegenus *Succinivibrio* (*Proteobacteria* phylum)betweenthetwosamplesgroups.Inaddition,inagreementwithourdata,theproportionoftheraregenus *Succinivibrio*,wasalsofoundconsiderablyhighinthestoolofJapanesepatientstreatedwithART[53].Oneofthepossiblereasonsforthecontradictoryresultsreportedintheexamineddifferentstudiesmayincludethecross-sectionalnatureofthestudy,theusedsamplingmethod(stoolswab *vs* stool),andthemicrobialtaxonlevelapplied.

Basedonourfindings,the24wkofARTinhibitedHIV-1viralreplicationeffectively(indeed,allenrolledpatientsreachedviralsuppression),butdidnotheavilyaffecttheoverallbacterialcompositionofthegutmicroenvironment.ThemodestGMdiversitythatweobservedbetweenthetwosamplegroupsmightbeassociatedwiththeloweringofviremia.However,therewasevidencethatARTalsoinduceschangesinthegutmicrobiome,unrelatedtoHIVinfection.SomeauthorshaveimpliedthatARTmayenhancedysbiosis,whichisconsistentwiththehighfrequencyofgastrointestinalsideeffectsofthistreatment[28,54].

AstheGMinfluencestheimmunesystemthroughtheirbacterialmetabolites,likeSCFAs[55,56]*,* wemeasuredSCFAlevelsinbloodandstoolsamples,inordertohaveamoreaccurateassessmentofmicrobialmetabolismaftertheART.Asknown,themainSCFAsinclude,inorderofproportion,acetic,propionic,andbutyricacidsthatareproducedbyfibresfermentationbygutbacteria,particularlybymembersofthe *Firmicutes* phylum[57].Interestingly,forthefirsttime,weobservedasignificantchangeoftwoserumSCFAsaftertheART.Inparticular,propionicandbutyricacidswereincreasedin“viralsuppression”condition.ThisalteredSCFAprofilemayindicateapotentialrolefortheSCFA synthesispathwayintheregulationoftheHIV“microbiota-immunity”axisduringeffectiveART.Notably,wedidnotobserveanysignificantSCFAchangesinstoolsamples,probablybecauseinthecolon,about95%oftheproducedSCFAsarerapidlyabsorbedbylargeintestinalmucosalcellswhiletheremaining5%aresecretedinthefeces[58].Propionateisonlypresentat alowconcentrationintheperipherybecauseitismetabolizedintheliver[59].IthasbeenshownthatbutyratemayreducegutinflammationbyinducingtheregulatoryTcells(Tregs)andmodulatingactivationofantigen-presentingcells[17].Wemayspeculatethatbacterialflorarespondsreciprocallytoinflammationbyincreasingthebiosynthesisofanti-inflammatoryandpro-solvinglipidmediatorsthatcirculateinthebloodstream.Altogether,itisplausiblethatimmunesystem-bacteriasynergismmediatessolutionstoinflammation.Onthecontrary,aspreviouslyreported,somestudieshavefoundthatbutyrate-producingbacteriaareselectivelyreducedinstoolsamplesfromHIV-infectedcomparedtonon-infectedsubjects[17,54].Inparticular,Serrano-Villar *et al*[60]foundthatHIV-infectedindividualshadadistinctSCFAprofileinstoolcomparedtoHIV-negativecontrols,withincreasedpropionateandlowerlevelsofacetate.NodatafromtheliteratureareavailableregardingSCFAlevelsinHIV+serumsamples,exceptastudyofSegal *et al*[61]reportingthathighervaluesofserumSCFAs,inconsequenceofanincreasedabundanceofpulmonaryanaerobicbacteriainHIV+patientsonART,inhibitedtheimmuneresponseto *M. tuberculosis*,likelyenhancingtuberculosissusceptibility.Theyobservedthatbaselineserumbutyrateandpropionatewereassociatedwiththesubsequentincreasinghazardoftuberculosis.Moreover,wealsoevaluatedserumFFAcompositionbeforeandafterARTtreatment.Indeed,increasedlevelsofFFAandproinflammatorycytokineshavebeenreportedinsomeHIV-infectedpatientsunderART(reviewedinreference[62]).However,wedidnotappreciateanydifferenceattheexaminedtwotimepoints.

Regardingtheinflammationtone,thereisconsensusthatapro-inflammatorystatusremainsactiveevenafterARTinitiationinmostpatients[63,64].SincetheHIVlifecycleissuppressedthroughARTintreatedpatients,thechronicinflammatorystatusobservedinpatientsismaintainedbyfactorssecondarytoHIVreplication,includingmicrobialtranslocationandreducedimmunoregulatoryfunction.InordertoevaluatetheinflammatorystatusafterART,wemeasuredapanelofselectedmultifunctionaleffectormoleculesoftheimmuneresponseinserum.Amongthemeasuredcytokines,weobservedadecreaseofIP-10(*P* =0.0244)afterthetreatment,confirmingthedownregulationofthischemokineproductioninpatientswithHIV infection duringART[65-69].IP-10isinvolvedintraffickingimmunecellstoinflammatorysites, anditisconsideredanimportantpro-inflammatoryfactorintheHIVdiseaseprocess.Ithasbeenobservedthatitslevelscanbereduced,butnottonormallevels,byARTadministration.Interestingly,IP-10wasconsistentlyassociatedwithHIV[diseaseprogression](https://www.sciencedirect.com/topics/medicine-and-dentistry/disease-exacerbation)(basedonCD4+counts)duringtheperiod[70],suggestingitspotentialforuseasanindicatorofHIVinfectionand/oratherapeutictargetforHIVtreatment[71].Ontheotherhand,inagreementwithrecentdata,weobservedasignificantincreasedtrendofIL-8levels(*P* =0.0547)withsuppressedviralloadafter24wkofART.Indeed,increasedIL-8levelswereobservedinHIV-infectedindividualsonART[72].IthasbeenshownthatduringHIV-1infection,IL-8playsanimportantroleintherecruitmentofCD4+Tcellstothelymphnodes,thusgeneratingmoretargetsforviralreplication.OurresultsmaysuggestthatincreasedIL-8LevelsmayrepresentahallmarkofchronicinflammationinHIV+patientsonART.Inaccordancewithourfindings,Wada *et al*[73]observedsignificantlyhighercirculatingIL-8levelsinHIV+menonARTwithsuppressedviralloadincomparisontoHIV-uninfectedmen.

ItisnowestablishedthatthegutmicrobiomemayplayacrucialroleintheimmuneactivationinHIV-infectedpatientstreatedwithART[5,64,73-75].Recently,severalstudieshavereportedthatGMisassociatedwithCD4+TcellrecoveryinHIV-infectedpatients,playinganessentialroleinthereconstitutionofimmunefunction[76-78].Thepotentialmechanismincludestheformationofavirusshelter,resistancetoART,promotionofintestinalmucosalbarrierdamage,andfurtherentryofintestinalbacteria andtheirmetabolitesintothecirculatorysystem,resultinginlong-termimmuneactivation,inflammation,andmetabolicdisorderssuchascardiovasculardiseases,diabetesmellitus,liversteatosis,andlastly,cancer[8].AlthoughitremainsunclearwhetheranalteredimmunityafterHIVinfectiondrivesdysbiosisor *vice versa*,thegutdysbiosis,immunedysfunction,epithelialdamage,andmicrobialtranslocationarestillevidenteveninthesettingofART-mediatedviralsuppression,whichmightbethetreatmentdilemmaforHIVinfectionatpresent.DespitenumerousstudiesofthemicrobiotainHIV-infectedpatients,therearerelativelyfewreportsdiscussingthecompositionalGMchangesinpatientswithdifferentimmuneresponsestoART[79,80].

Toinvestigatethe role ofGM inimmunomodulationandimmunereconstitutionandwhichbacterialmetabolitesareimplicated,inthesecondpartofthestudy,wedividedthepatientsintotwogroups:PatientswithCD4/CD4ratio< 1withinsufficientreconstitutionofCD4+Tcellsdespiteachievingvirologicalsuppressionafter24wkofARTandthosewithCD4/CD8≥ 1whoreachedarobustreconstitutionofCD4+Tcells.Wefoundthatthe *Anaerostipes* genuswassignificantlyaugmentedinIRs;onthecontrary,the *Faecalibacterium* genuswassignificantlyincreasedinINRs.Notably,*Faecalibacterium* hasbeenreportedastheanti-inflammatorycommensalgenus[81].IthasbeenpositivelycorrelatedwiththeCD4/CD8ratioandanti-correlatedwithinflammationmarkersandLPSinarecentstudyinHIV-infectedpatients[82].

Regardingmicrobialmetabolites,wedetected asignificantincreaseinfecalisobutyric,isovaleric,and2-methylbutyricacidsintheIRs.However,wefoundthatthechangesassociatedwiththeIRgroupwerenotevidentintheblood.Basedonourresults,wehypothesizedthatchangesatthegenuslevelinthegutecosysteminHIV-infectedpatientsundergoingARTmightthusbebothaconsequenceandapotentialcauseoftherecoveryofsystemicimmunity.

Ourstudyhadsomelimitations.First,alownumberofpatientswereenrolledtoinvestigatetheelementsofthemicrobiota-immunityaxisanditcannotdeterminewhetherthealteredGMcontributedtoorwascausedbyimmunedysfunction.Second,onlytheeffectsof24*-*wkARTwereobservedinourstudy,andtoestablishamoremeaningfulconnectionbetweenGMandmicrobial/immuneparameters,futurestudiesshouldinvestigatetheGMalterationsandtherestorationofimmunefunctionafterlong-termeffectiveART.Finally,themicrobiotaoffeceswasaproxyforGMinthisstudy,whichwastheonlyrealisticsampleforanon-invasivestudy.However,fecalmicrobiotamayonlyrepresenttheGMcompositioninthelumenratherthanonthemucosalsurfaces,whichisanimportantdistinctionbecausethemucosa-associatedmicrobiotapotentiallyinteractswiththegut-associatedlymphoidtissueinHIV-1-infectedpatientsdirectly.

**CONCLUSION**

OurresultsprovidedanadditionalvisionabouttheimpactofHIVinfection,art,andimmunerecoveryinthemicrobiota-immunityaxisatthemetabolismlevel,whichareanindicatoroftheactiveprocessesoccurringinthegastrointestinaltract.Insummary,wedemonstratedthatpatientsinfectedbyHIV-1,afterreachingvirologicalsuppressionwithART,displayedafecalmicrobiotawithunchangedoverallbacterialdiversityexceptforfewgenera.Although24wkoftreatmentwithARTwaseffective,thesystemicinflammatorytonewasnotcompletelyrestoreddespitetheanti-inflammatoryserumbutyrateincrement.Inaddition,weconfirmedtheroleoftheGMinimmunereconstitution,withthepossibleimplicationofbacterialmetabolites;however,changesinthegutecosysteminHIV+patientsundergoing24wk ofARTmaythusbebothaconsequenceandapotentialcauseoftherecoveryofsystemicimmunity.

Futurelarger-scale,long-termARTandlongitudinalstudiesthatincludefunctionalmetagenomicandmetabolomicapproachestoidentifytherolesofthespecificdifferentialphylotypesarerequiredtobetterdefinetherelationshipbetweenmicrobiota-immunityaxisandHIV-1infectionandtoprovidenewinsightsintothetargetedtreatment,improvingtheimmunerecoveryanddampening inflammation.

**ARTICLEHIGHLIGHTS**

***Research background***

Human immunodeficiency virus type 1 (HIV-1)infectionischaracterizedbypersistentsystemicinflammationandimmuneactivation,eveninpatientsreceivingeffectiveantiretroviraltherapy(ART).Convergingdatasuggestthat gutmicrobiota(GM)changescanoccurthroughoutincludinghumanimmunodeficiencyvirus(HIV)infectiontreatedbyART.

***Research motivation***

ARThasincreasedthelifeexpectancyofHIV-infectedpatients;however,chronicinflammationandgutmicrobiotaalterationspersistinpatientsvirologicallysuppressedbyART.Thesedatasuggestthatre-shapingthemicrobiotamaybeanadjuvanttherapyinpatientscommencingsuccessfulART

***Research objectives***

Thepurposeofthisprospectiveobservationalstudywastocompareforthefirsttimethefecalmicrobialcomposition,serumandfecalmicrobialmetabolites,andserumcytokineprofileoftreatment*-*naïvepatientsbeforestartingARTandafterreachingvirologicalsuppression(HIVRNA<50copies/mL)after24wkofART.

***Research methods***

The authorsenrolled12treatment*-*naïve HIV-infectedpatientsreceivingART.Fecalmicrobiotacompositionwasassessedthroughnextgenerationsequencing,andacomprehensiveanalysisofabroad spectrum ofcytokines in blood wasperformedthroughamultiplexapproach.Inaddition,serumfreefattyacid(FFA)andfecalshortchainfattyacid(SCFA)levelsweremeasuredthroughGC-MS.

***Research results***

The authorscomparedmicrobiotasignatures,FFAlevels,andcytokineprofilebeforestartingARTandafterreachingvirologicalsuppression.Modestalterationswere observedonmicrobiotacomposition;moreover,inthesamecondition,wealsoobservedaugmentedlevelsofserumpropionicandbutyricacids.AreductionofserumIP-10andanincreaseofIL-8levelweredetectedin theviralsuppressioncondition.Thereafter,thesamecomponentswerecomparedbetweenimmunologicalrespondersandnon-responders.Concerningthemicroflorapopulation,wedetectedareductionof *Faecalibacterium* andanincreaseof *Alistipes* inimmunologicalnon-responders.Simultaneously,fecalisobutyric,isovaleric,and2-methylbutyricacidswerealsoincreasedinimmunologicalnon-responders.

***Research conclusions***

TheresultsprovidanadditionalperspectiveabouttheimpactofHIVinfection,ART,andimmunerecoveryonthe“microbiome-immunityaxis”atthemetabolismlevel.Thesefactorscanactasindicatorsoftheactiveprocessesoccurringinthegastrointestinaltract.

***Research perspectives***

Futurelarger-scale,long-termARTandlongitudinalstudiesthatincludefunctionalmetagenomicandmetabolomicapproachestoidentifytherolesofthespecificdifferentialphylotypesarerequiredtobetterdefinetherelationshipbetweenmicrobiota-immunityaxisandHIV-1infectionandtoprovidenewinsightsintothetargetedtreatment,improvingtheimmunerecoveryanddampeninginflammation.

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**Footnotes**

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**FigureLegends**



**Figure1Box-plotsshowingalphadiversityindices(Chao1,Shannon, andevenness indices)insamples.**StatisticaldifferenceswereevaluatedusingpairedWilcoxonsigned-ranktestforChao,Shannon,andevennessindices. *P* valuelessthan0.05wereconsideredstatisticallysignificant.



A B

**Figure2Clusteranalysis(a)andprincipalcoordinateanalysisshowingthatsamplesdonotseparateintotwogroupsdependingontheircondition(0-24wk) (B).**



**Figure3Segmentplotsdepictingtaxawithsignificantlydifferencesbetweenhighviremia(time point0)andviralsuppression(time point24)conditions.**Linesconnectpairedsamplesandhighlightthedifferencesinnormalizedabundancefortheindicatedrank.Orangeorbluecolorshighlightdecreaseorincrease,respectively.Numbersinthetop-leftcornerrepresentcountsofincreased(orange)anddecreased(blue)measurementforpairedsamples.

*P* < 0.05

*P* < 0.05

**Figure4Boxplotsshowing statisticallydifferentlevelsofserumshort-chain fatty acids betweenhighviremiaandviralsuppressorpatients,assessedbytheWilcoxontest.** *p* value<0.05wasconsideredstatisticallysignificant.

*P* < 0.05

*P* < 0.05

**Figure5Boxplotsshowing statisticallydifferentlevelsofserumcytokinesbetweenhighviremiaandviralsuppressorpatients,assessedbytheWilcoxontest.**A *p* value<0.05wasconsideredstatisticallysignificant.



**Figure6Boxplotsshowingtheresultsoftaxa-leveldifferentialabundanceanalysisbetweenimmunological respondersandimmunological non-respondersat 24wk.**PlottitlesreporttheshrunkLog2foldchange(accordingtotheDESeq2functionlfcShrink).Allresultshavea *p* value<0.05.NR = INRs, R = IRs. IRs: immunological responders; INRs: immunological non-responders.



a

a

a

**Figure7Boxplotsshowing statisticallydifferentfecalshort-chain fatty acidabundancesbetweenimmunological responders andimmunological non-responders,assessedbytheMann-Whitneytest.**a*p* value < 0.05wasconsideredstatisticallysignificant.

**Table 1 Features of the enrolled patients**

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Age** | **Sex** | **ART regimen** | **Comorbidities** | **Timepoints (wk)** | **Viral load (copies/mL)** | **CD4+ cells/mm3** | **CD8+ cells/mm3** | **CD4/CD8 ratio** |
| 1 | 37 | Male | 3TC/ABC/DTG | No | T0 | 597463 | 110 | 420 | 0.3 |
| T24 | < 20 | 520 | 832 | 0.6 |
| 2 | 38 | Male | FTC/TDF/EVG/C | No | T0 | 4489 | 630 | 670 | 0.9 |
| T24 | TND | 831 | 740 | 1.1 |
| 3 | 34 | Male | FTC/TDF/EVG/C | No | T0 | 165516 | 253 | 725 | 0.3 |
| T24 | TND | 504 | 363 | 1.4 |
| 4 | 39 | Male | FTC/TDF/EVG/c | No | T0 | 859883 | 360 | 974 | 0.4 |
| T24 | 33 | 781 | 986 | 0.8 |
| 5 | 38 | Male | 3TC/ABC/DTG | No | T0 | 4860 | 1341 | 928 | 1.4 |
| T24 | TND | 1881 | 988 | 1.9 |
| 6 | 41 | Male | FTC/TDF/RPV | Atrial fibrillation | T0 | 213 | 814 | 690 | 1.2 |
| T24 | TND | 845 | 519 | 1.6 |
| 7 | 25 | Male | 3TC/ABC/DTG | No | T0 | 23098 | 516 | 1149 | 0.4 |
| T24 | < 20 | 942 | 1019 | 0.9 |
| 8 | 22 | Male | FTC/TAF/EVG/c | No | T0 | 12188 | 654 | 1055 | 0.6 |
| T24 | TND | 668 | 733 | 0.9 |
| 9 | 48 | Male | 3TC/ABC/DTG | No | T0 | 175 | 833 | 1520 | 0.5 |
| T24 | TND | 941 | 1258 | 0.7 |
| 10 | 53 | Male | 3TC/ABC/DTG | Hypertension, HCV | T0 | 40545 | 863 | 1196 | 0.7 |
| T24 | TND | 612 | 515 | 1.2 |
| 11 | 40 | Male | 3TC/ABC/DTG | No | T0 | 859000 | 399 | 980 | 0.4 |
| T24 | 39 | 648 | 652 | 1 |
| 12 | 51 | Male | FTC/TDF DTG | Diabetes  | T0 | 4410 | 884 | 1066 | 0.8 |
| T24 | < 20  | 1130 | 1261 | 0.9 |

ART: Antiretroviral therapy; 3TC: Lamivudine; ABC: Abacavir; DTG: Dolutegravir; FTC: Emtricitabine; TDF: Tenovir; EVG/c: Elvitegravir/cobi; RPV: Rilpivirine.