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Impaired gut microbiota-mediated short-chain fatty acid production precedes morbidity and mortality in people with HIV

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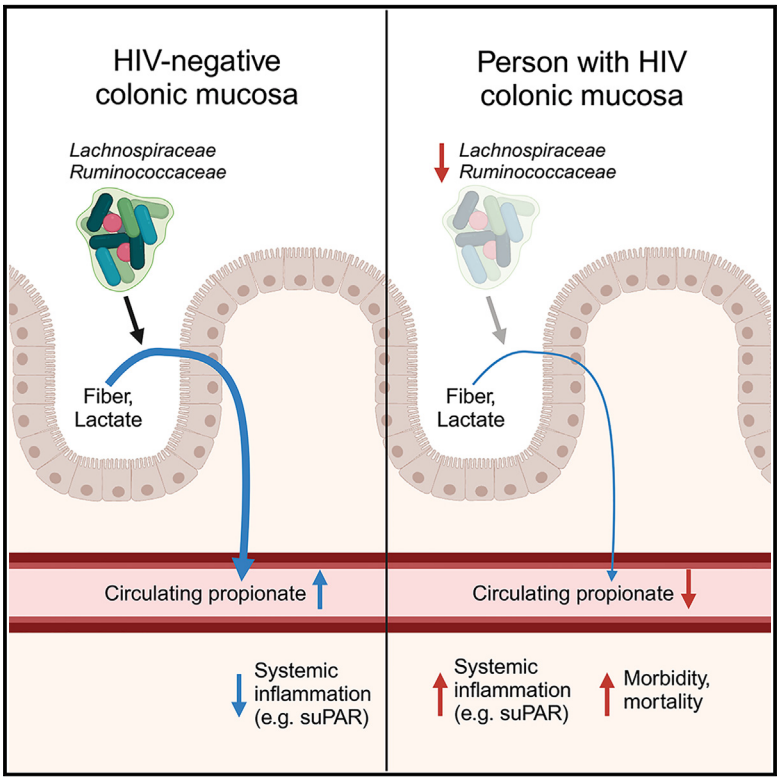
Graphical abstract



Highlights

- Gut microbiota propionate production is better represented in serum than feces
- People with HIV exhibit loss of microbiota-encoded SCFA production
- Systemic SCFAs associate inversely with inflammation in people with HIV
- Failure to convert lactate to propionate precedes death and comorbid events in PWH

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In brief

Sereti et al. examine microbiota composition and short-chain fatty acid (SCFA) dynamics in serum and stool of people with HIV (PWH) sampled prior to onset of comorbidities or death. Systemic SCFAs are significantly depleted in PWH and are associated with prognostic inflammatory markers, and decreases in gut microbial SCFA production pathways precede comorbidities and death in PWH.

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Impaired gut microbiota-mediated short-chain fatty acid production precedes morbidity and mortality in people with HIV

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SUMMARY

Antiretroviral therapy (ART) has dramatically lengthened lifespan among people with HIV (PWH), but this population experiences heightened rates of inflammation-related comorbidities. HIV-associated inflammation is linked with an altered microbiome; whether such alterations precede inflammation-related comorbidities or occur as their consequence remains unknown. We find that ART-treated PWH exhibit depletion of gut-resident bacteria that produce short-chain fatty acids (SCFAs)—crucial microbial metabolites with anti-inflammatory properties. Prior reports establish that fecal SCFA concentrations are not depleted in PWH. We find that gut-microbiota-mediated SCFA production capacity is better reflected in serum than in feces and that PWH exhibit reduced serum SCFA, which associates with inflammatory markers. Leveraging stool and serum samples collected prior to comorbidity onset, we find that HIV-specific microbiome alterations precede morbidity and mortality in ART-treated PWH. Among these microbiome alterations, reduced microbiome-mediated conversion of lactate to propionate precedes mortality in PWH. Thus, gut microbial fiber/lactate conversion to SCFAs may modulate HIV-associated comorbidity risk.

INTRODUCTION

The advent of potent combination antiretroviral therapy (ART) radically changed the face of the AIDS epidemic, transforming treated HIV infection into a chronic disease.[1] Development of new, less toxic antiretroviral medications with more convenient dosing over the past decade, combined with earlier ART initiation at higher CD4 cell counts, has further improved virologic suppression and overall survival.[2–4] Although closing, a gap in survival between people with HIV (PWH) and the general population continues to persist,[5,6] driven by an increased risk in PWH of non-AIDS comorbidities such as cardiovascular disease, non-AIDS-defining malignancies, and chronic kidney disease, among others.[7–9] Chronic inflammation has been the thread that ties many of these comorbidities to both HIV and aging.[10,11]

The pathogenesis of HIV-associated inflammation is incompletely understood but has been linked to an altered gut microbiota composition,[12] namely reductions in alpha diversity and a loss of gut bacteria capable of producing short-chain fatty acids (SCFAs) from dietary fibers.[13–17] Murine studies highlight SCFAs (specifically butyrate and propionate) as having critical beneficial effects on host physiology relevant to HIV-associated comorbidities including dampening inflammation, augmenting gut barrier function, and conferring protection from cardiovascular disease.[18–25] While PWH exhibit a loss of gut commensal bacteria thought to be important SCFA producers,[26,27] whether

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bioavailability of SCFA is indeed reduced in PWH remains controversial. Prior studies examining abundance of SCFAs in feces have reported minimal differences between PWH and HIV-negative groups and/or mixed results.[28–31] This apparent paradox of a lack of clear reduction of SCFA fecal content despite a reduced abundance of SCFA-producing gut microbes remains an unresolved question. Furthermore, whether microbiota alterations precede clinical manifestations of inflammation-related comorbidities in treated PWH—and are thus less likely to be consequences of a comorbidity—remains poorly defined.

Regularly sampling humans longitudinally and capturing samples prior to onset of pathologic events is a powerful strategy to trace potential causal effects of the microbiota on future incidence of comorbidities. Utilizing a large prospective cohort of longitudinally followed ART-treated PWH, we selected fecal and serum samples obtained at a single time point per individual that preceded the onset of several common HIV-associated comorbidities and death. Comorbidities examined included chronic kidney disease, cardiovascular disease, and cancer, comprising the predominant comorbidities that treated PWH are at greater risk of developing as compared to people without HIV.[7–9] This longitudinal design was employed to explore how microbiome patterns—and alterations in SCFA production capacity—affect the risk of development of future incident non-AIDS inflammatory comorbidities and death in PWH.

RESULTS

Study population

Of 922 total individuals within the AGE_hIV cohort that had stool collected, we selected 138 total participants (53 cases and 85 control subjects) that either had an incident event of interest (case group)—comorbidity or death—or that did not experience either (control group) (Figure S1). Incident comorbidities of interest within this population included cardiovascular disease (n = 13 cases), cancer (n = 13 cases), and chronic kidney disease (n = 6 cases), which were chosen for being among the most prevalent sources of morbidity in chronic, ART-treated PWH.[7,32] Control subjects were matched to cases (2 control subjects per 1 case for incident comorbidities and 1:1 for death) based on the following: HIV status, sex at birth, age, BMI, birth country, sexual practice, receptive anal intercourse in the past 6 months, smoking pack years, current smoking status, and current alcohol consumption (details in STAR Methods). Cases and control subjects exhibited comparable lengths of time on ART (Tables S1 and S3).

From this cohort, which included both PWH and participants without HIV, our primary analyses focused on PWH (n = 38 cases of morbidity/mortality of PWH and n = 57 matched control PWH without morbidity/mortality). For PWH who went on to develop a comorbidity (n = 19; Tables S1 and S3), stool samples were selected that preceded comorbidity development, and the median length of time between sampling and clinical diagnosis of each comorbid event was 405 days before the clinical event (interquartile range [IQR] 239–601 days before events). Stool sampling for PWH who died was a median of 498 days preceding mortality (IQR: 317–905 days; n = 19 cases, n = 19 control subjects; Table S4). Sera and plasma from all study participants from near the time of stool sampling were also analyzed and had a median of 0 days from stool sampling (IQR: −1 to 0).

To identify features that differed between PWH and participants without HIV, a subpopulation of PWH and an HIV-negative group were selected (inclusive of all participants regardless of comorbidity or mortality case/control status) that were matched for relevant confounding variables by stratification of participants by sex, sexual behavior, age, BMI, and alcohol consumption (outlined in STAR Methods). Detailed characteristics of this resulting subpopulation (n = 66 PWH and n = 36 HIV-negative participants) are found in Table S5.

Evaluation of antecedent soluble biomarkers of inflammation in relation to incident clinical events in PWH

We sought to evaluate soluble biomarkers between PWH with a future incident event (cases) and without an incident event (control subjects) from plasma obtained prior to events and proximal to stool sampling. We selected biomarkers that quantify inflammation and complement activation, which we had found to associate with microbiota compositional features in a previous cross-sectional analysis within the AGE_hIV cohort study.[14,33] As compared to control subjects, cases exhibited significantly higher levels of circulating soluble urokinase plasminogen receptor (suPAR; p = 0.0376; Figure 1A), an inflammatory biomarker that is predictive of cardiovascular morbidity and death in case-control studies of people with and without HIV.[34,35] In addition, complement component 5 (C5) was higher in cases compared to control subjects (p = 0.0216, Figure S2). These observations are concordant with prior studies[34,35] and suggest that our small cohort was sufficiently powered to recapitulate and validate established findings.

Distinct microbiome patterns precede incident clinical events in PWH

Prior studies have linked specific microbiota composition patterns with HIV status and markers of inflammation and disease progression,[15,36,37] though whether such microbiome patterns precede clinical comorbid events is unclear. To collapse the microbiota changes that independently associated with chronic HIV infection into a single variable, we calculated an "HIV-associated dysbiosis index" (HADI; see STAR Methods). A higher HADI, indicative of greater extent of HIV-associated dysbiosis and ecological distance from HIV-negative microbiome profiles, was associated with higher levels of suPAR when examining all PWH (p = 0.0061; Figure 1B). A higher HADI was also observed in the 13 PWH who developed incident cardiovascular disease (CVD; i.e., cerebrovascular accident [CVA], myocardial infarction, or angina pectoris) compared to their matched control subjects (p = 0.0296; Figure 1C). For non-AIDS-defining cancers, chronic kidney disease (CKD) and death, there were no significant differences between case PWH and control subjects (p = 0.1744, 0.7016, and 0.3870, respectively).

Alpha diversity has been reported as being reduced in the microbiota of PWH.[13–16] We found concordant results when comparing alpha diversity in the gut microbiota between PWH and HIV-negative participants from the selected subgroup (combining cases with an incident event and control subjects

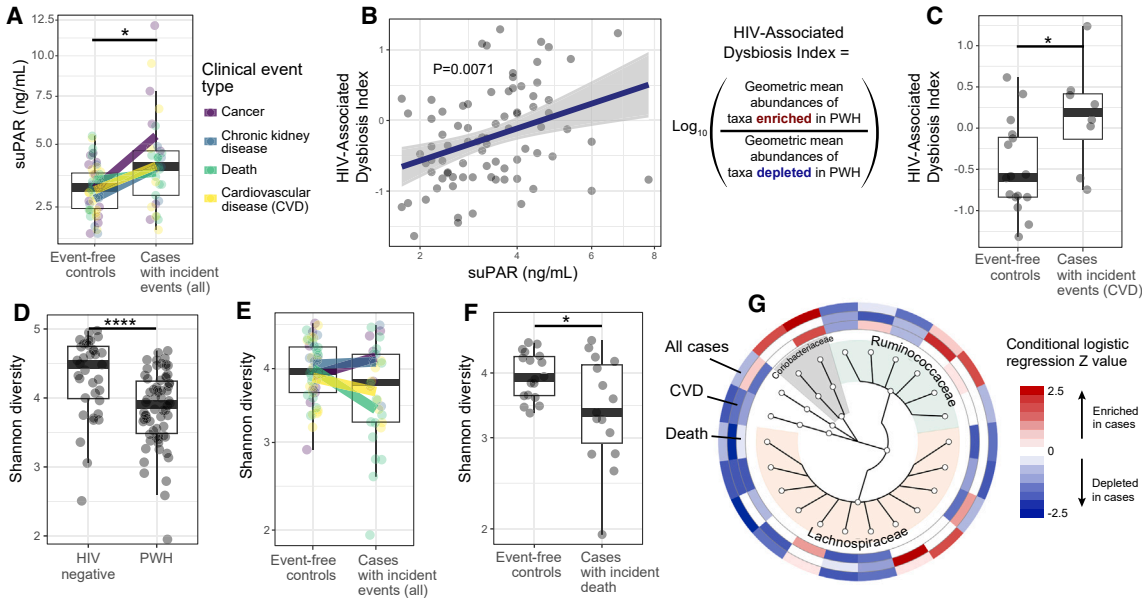

Figure 1. Fecal microbiome composition patterns associate with prognostic biomarkers of inflammation and incidence of death and co-morbid events in PWH

(A) Soluble urokinase plasminogen receptor (suPAR) measured in plasma is higher in incident event individuals with HIV compared to event-free control subjects with HIV (p = 0.0376, conditional logistic regression, n = 33 cases, n = 51 control subjects).

(B) HIV-associated dysbiosis index (HADI; defined in image) correlates with plasma suPAR (p = 0.0061, linear mixed-effects model controlling for age, BMI, and sex) within PWH (n = 81).

(C) HADI is elevated in cases of cardiovascular disease (CVD) with HIV compared to non-CVD control subjects with HIV (p = 0.0423, conditional logistic regression, n = 8 cases, n = 16 control subjects).

(D) Shannon alpha diversity is decreased in PWH compared to HIV-negative persons (p = 0.000034, conditional logistic regression, n = 66 PWH and n = 36 HIV-negative subgroups).

(E) Shannon alpha diversity trends toward a decrease when comparing all incident cases with HIV to event-free control subjects with HIV (p = 0.0611, conditional logistic regression, n = 32 affected individuals and n = 47 control subjects). Legend visible in (A).

(F) Shannon alpha diversity is decreased in participants with HIV who died compared to those with HIV who did not die (p = 0.0375, conditional logistic regression, n = 15 cases and n = 18 control subjects).

(G) Amplicon sequence variants (ASVs) that differed in abundance between PWH and control subjects for the categories of death, CVD, and all cases considered together (i.e., cancer, chronic kidney disease, death, and CVD). ASVs with p <0.02 in any of the three aforementioned categories by conditional logistic regression are shown. Colors indicate Z regression values. Axes are shown in square-root format. *p < 0.05, ****p < 0.0005. For all boxplots: center line, median; box limits, upper and lower quartiles; whiskers, 1.5× interquartile range.

without events), stratified for sex, sexual behavior, age, BMI, and alcohol consumption ($p = 3.4*10^{-5}$; Figure 1D). We next compared alpha diversity between all PWH cases and PWH control subjects. We observed a non-significantly lower Shannon diversity in PWH who went on to experience any event (death or comorbidities) versus their control subjects (p = 0.0611; Figure 1E). In PWH who later died, Shannon diversity was significantly lower (p = 0.0375; Figure 1F) compared to their matched control subjects. These findings were not evident in cases without HIV compared to their matched control subjects (p = 0.5120 and 0.5730, respectively). To identify specific gut bacterial taxa associated with eventual onset of comorbid events, we compared abundances of all 16S amplicon sequence variants that differed between (1) all PWH cases (n = 38, any comorbidity or death) and their matched PWH control subjects (n = 57); (2) PWH who died (n = 19) and their matched PWH control subjects (n = 19); and (3) PWH who developed CVD (n = 9) and their control subjects (n = 18). We found that abundances of several *Lachnospiraceae* and *Ruminococcaceae* taxa were depleted in all cases, cases who developed CVD, and cases who died compared to their matched control subjects (Figure 1G).

Gut microbial taxa involved in SCFA production are decreased in PWH

We performed a meta-analysis aggregating 16S rRNA profiling data from six independent studies[16,36–40] (n = 330 total subjects across all six studies; n = 200 PWH and n = 130 HIV-negative individuals) and found that *Lachnospiraceae* and *Ruminococcaceae* were the most robustly decreased bacterial families in relative abundance among PWH compared to HIV-negative participants across all six studies queried (Figure 2A). These two families of human gut commensal microbes comprise major producers of SCFAs, microbial metabolites of fiber degradation that are critical for colonic epithelial cell energy homeostasis,[41–43] barrier function,[44] induction of inflammation-dampening T regulatory cells,[18–20] and induction of the secretion of the mucosal-barrier-protective cytokine interleukin-22 (IL-22).[24,25] The aforementioned meta-analysis included studies in which the important

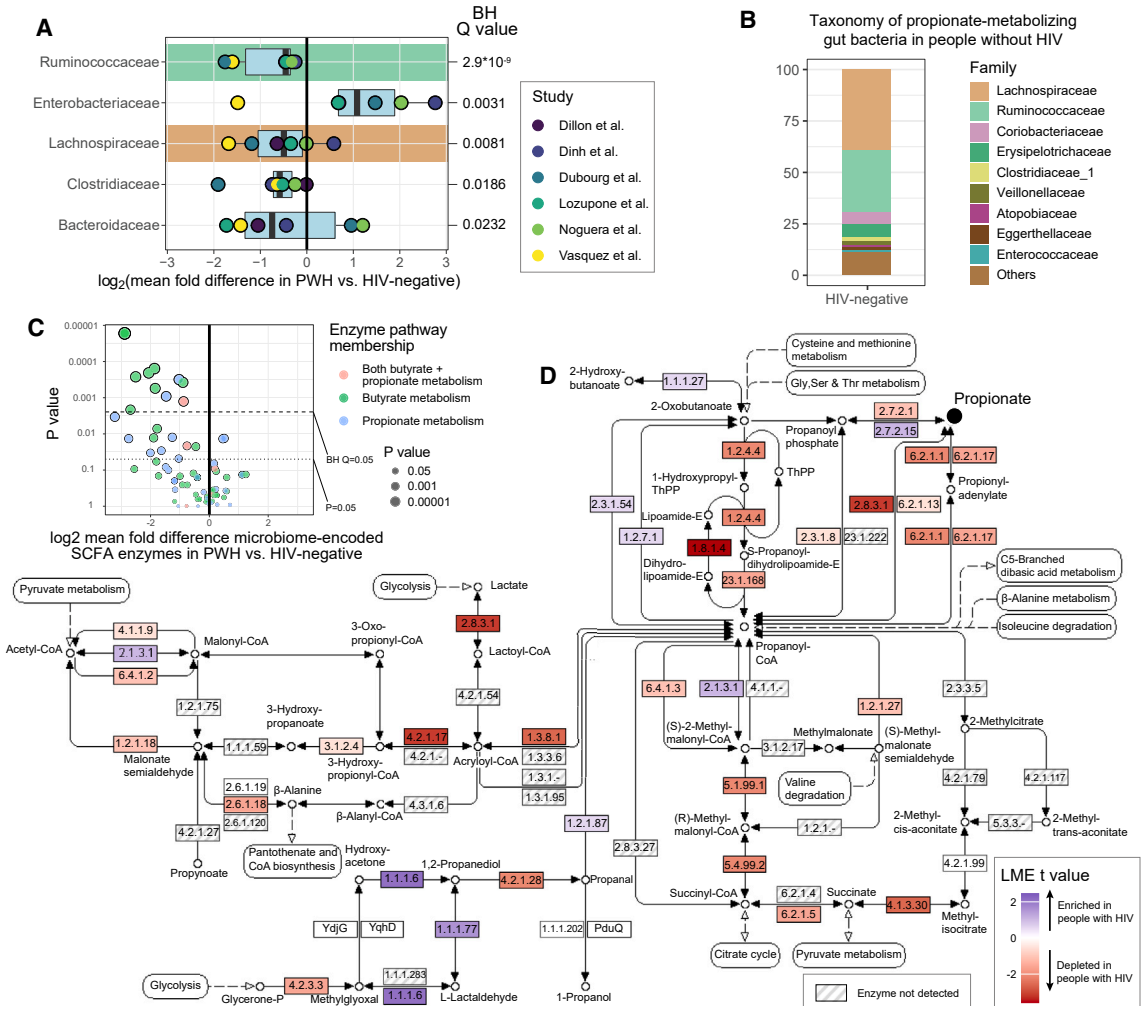

Figure 2. Propionate-producing gut bacteria and metabolic machinery are depleted in PWH

(A) Meta-analysis of 16S microbiota profiling data from six independent studies (n = 330 total individuals) examining microbiota differences between PWH versus people without HIV. Linear mixed models controlling for study, sample type, and intrastudy geographical locations were performed for all bacterial families.

(B) Taxonomic composition of propionate-production machinery in the HIV-negative group. Enzyme abundances were imputed from 16S data and taxonomic classifications of relative abundance contributions to each enzyme commission (EC) classification were obtained using PICRUSt2. ECs present in more than one participant and annotated as part of the propionate production pathway (KEGG pathway ko00640) were selected (n = 35 ECs). Family level relative contributions to total microbiota-encoded propionate production were summarized across all 35 ECs and all study participants.

(C) Enzymes involved in propionate and/or butyrate production (ECs belonging to ko00650 or ko00640) exhibit depletion in the gut microbiota of PWH. Linear mixed models controlling for age were used to test differences in EC relative abundances between people with and without HIV (n = 66 PWH and n = 35 HIV-negative individuals).

(D) KEGG pathway ko00640 for propionate (propanoate) metabolism. Colors denote linear mixed-effects t values, showing extent of depletion (red) or enrichment (blue) of each enzyme in PWH. BH, Benjamini-Hochberg false discovery calculations.

For (A): center line, median; box limits, upper and lower quartiles; whiskers, 1.5× interquartile range.

confounding variable of sexual behavior was not tracked, and thus subsequent analyses focused on our cohort of sample individuals from within the AGE$_h$IV cohort study, which are matched for sexual behavior, age, BMI, sex, and alcohol consumption. In the HIV-negative subgroup within the AGE$_h$IV study population (Table S5), the dominant taxonomic source of enzymes involved in production of the SCFA propionate originated from the *Lachnospiraceae* and *Ruminococcaceae* families (Figure 2B). When imputing microbiome function in our present cohort, we found that the enzymatic machinery for both propionate and butyrate production was significantly depleted in the PWH subgroup compared to the HIV-negative subgroup (gene set enrichment analysis [GSEA] $p = 0.026$ and $1.92*10^{-5}$ for propionate and butyrate enzymes based on KEGG pathways, respectively; Figures 2C and 2D). To investigate whether frequency of receptive anal intercourse influenced the observation of SCFA enzymatic machinery depletion in PWH, we examined PWH and HIV-negative groups that had reported no receptive anal intercourse in the past 6 months (Figure S3) and found concordant results in which SCFA machinery was depleted in PWH.

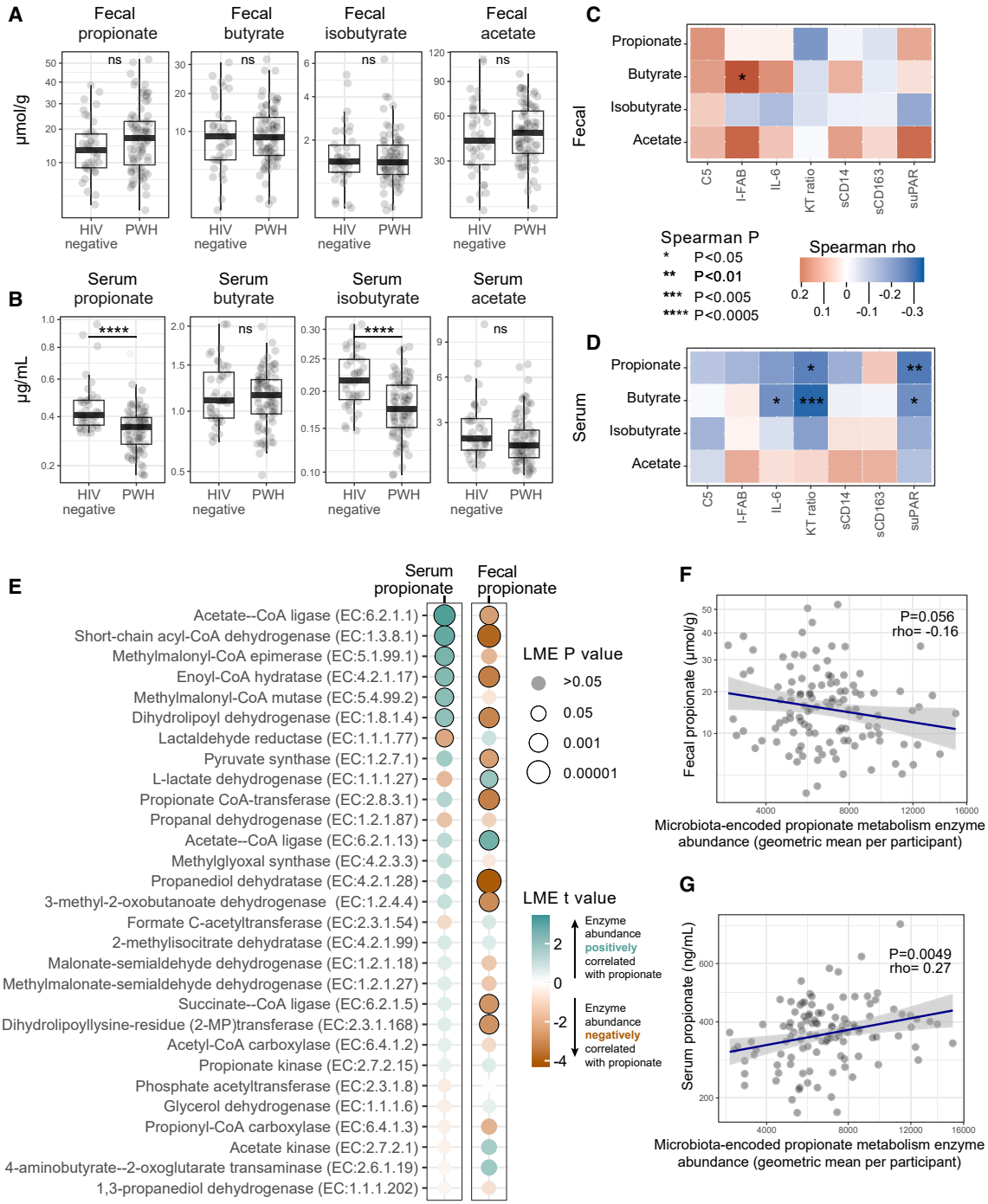

Figure 3. Propionate production by the gut microbiota is reflected in serum, not feces, and is reduced in PWH

(A) Concentrations of acetate, propionate, and butyrate in feces did not differ significantly between people with and without HIV (n = 37 HIV-negative individuals and n = 67 PWH).

(B) Serum concentration of propionate is significantly lower in PWH compared to the HIV-negative group (p = 0.000031, conditional logistic regression; n = 35 HIV-negative individuals and n = 63 PWH).

(C and D) Fecal (C) and serum (D) concentrations of butyrate, propionate, and acetate were compared to serological markers of inflammation (IL-6, KT ratio, sCD14, and suPAR) and gut barrier injury (I-FABP) using Spearman correlation tests within PWH (n = 90).

(E) Relative abundance of each enzyme involved in propionate production (KEGG pathway ko00640) was compared to either serum propionate concentrations (left column, n = 83 PWH and n = 40 HIV-negative individuals) or fecal propionate concentrations (right column, n = 82 PWH and n = 40 HIV-negative individuals) in

(legend continued on next page)

Microbial SCFA production is decreased in PWH and is better reflected in serum than in feces

Despite a robust loss of *Lachnospiraceae* and *Ruminococcaceae* as well as imputed enzymatic machinery for SCFA production, SCFAs measured in stool did not differ between the PWH and HIV-negative subgroups (Figure 3A; Table S6). Because SCFAs are actively taken up by transporter proteins expressed in the colonic epithelium for the purpose of being utilized by host cells in the gut and periphery for cellular energy,[45] abundance of SCFAs in excreted feces reflects the balance of microbiota-mediated SCFA production minus host-mediated uptake. By querying transcriptomic data within a previously published study,[46] we found that exposure of human colonic epithelial cells to SCFAs increased expression of SCFA transporters in a dose-dependent manner (Figure S4A), suggesting that SCFA uptake is dynamically regulated in response to luminal SCFA concentrations and thus that fecal SCFAs may not accurately reflect bioavailable SCFAs produced by the microbiota *in situ*. Indeed, if higher colonic luminal SCFA induces transporter expression and increased SCFA uptake, such a dynamic system may result in concentrations of excreted fecal SCFAs being similar between individuals with high and low SCFA production (Figures S4B and S4C). We therefore additionally measured SCFAs in the circulation, which is more likely to represent the uptaken SCFA pool. In accordance with the loss of propionate-producing gut microbes and microbial enzymes, we observed a significant depletion of propionate and isobutyrate in the serum of PWH as compared to the HIV-negative subgroup (Figure 3B). Despite a significant reduction in butyrate-producing enzymes in the gut microbiota of PWH (Figure 2C), butyrate was not observed to be depleted in serum or stool, possibly due to its previously observed near-complete uptake and preferential consumption by colonic epithelial cells.[47] Serum, but not fecal, propionate correlated inversely with prognostic markers of inflammation[34,48] (IL-6, KT ratio, sCD14, and suPAR) as well as gut barrier injury (I-FABP; Figures 3C and 3D). When comparing the abundances of gut microbial enzymes involved in propionate production to concentrations of propionate in feces and serum, we found that these enzymes correlated positively with serum but not fecal propionate ($p = 0.0049$ with rho = 0.27 and $p = 0.0566$ with rho = −0.16, respectively; Figures 3E–3G), suggesting that microbiota-mediated propionate production is more accurately reflected in serum than in feces.

Impaired gut microbial conversion of lactate to propionate occurs in PWH, correlates with inflammation, and precedes death

Complex carbohydrates in the form of dietary fiber are the predominant substrates of gut microbial SCFA fermentation. Lactate is a common byproduct of fiber fermentation[49] and is itself an intermediate in the conversion of fiber to SCFAs including propionate.[50–53] Indeed, conversion of lactate to propionate (Figure 4A) is one of the predominant enzymatic pathways for propionate production in the human gut microbiota.[51,54] We found that the gut bacterial enzymatic machinery to convert lactate to propionate was significantly depleted in PWH compared to the HIV-negative subgroup (Figure 4B). In accordance with a loss of lactate conversion to propionate, buildup of lactate was evident in PWH in the form of elevated fecal lactate compared to the HIV-negative subgroup ($p = 0.0082$; Figure 4C). In individuals with HIV who died, significantly elevated fecal lactate was observed as compared to their matched alive control subjects ($p = 0.0325$; Figure 4D), along with a significant depletion in the abundance of gut microbial enzymes involved in conversion of lactate to propionate ($p = 0.0385$; Figure 4E). Fecal lactate correlated inversely with Shannon diversity and positively with the HADI (Figures S4A and S4B), suggesting a link between fecal lactate and HIV-associated changes in gut microbiota composition. To understand whether flux through the pathway of luminal lactate conversion to propionate was associated with incident events and inflammatory markers, scaled ratios of fecal lactate to serum propionate were calculated. Fecal lactate-to-serum propionate ratios correlated with the prognostic inflammatory biomarker suPAR across all participants after adjusting for age, BMI, HIV serostatus, and sex ($p = 0.0067$; Figure 4F). This correlation was also significant when considering either fecal lactate or serum propionate alone ($p = 0.0154$ or 0.0182, respectively; Figures S5C and S5D). Fecal lactate-to-serum propionate ratios were also significantly higher when examining all cases with HIV (incident comorbidities and deaths) compared to control subjects with HIV ($p = 0.0262$; Figure 4G). This ratio was also more significant in this comparison than either fecal lactate or serum propionate alone ($p = 0.0299$ or 0.3100, respectively; Figures S5E and S5F).

DISCUSSION

In this study, we assessed the relationship between HIV-associated gut microbiome composition, gut microbial metabolites, systemic inflammation, and incidence of clinical events that relate to aging and inflammation in a cohort of older adults with and without HIV. The cases with incident events of interest and control subjects without such events were carefully matched, and sampling of stool and serum preceded clinical events, on average, by 1–2 years. As expected, inflammatory markers including suPAR were associated with onset of HIV-associated comorbidities. We find that PWH exhibit a loss of microbiome-encoded capacity to produce SCFAs including butyrate and propionate in the *Lachnospiraceae* and *Ruminococcaceae* families and that circulating concentrations of these SCFAs correlated

all participants with corresponding data. To control for HIV serostatus, linear mixed-effects tests were performed with HIV as a covariate. Significant associations ($p < 0.05$) are denoted by black outlined circles.

(F and G) Geometric mean relative abundances of all enzymes annotated as contributing to propionate production (ko00640) were calculated for participants with all three data types available (n = 35 HIV-negative individuals and n = 79 PWH). This summary measure of microbiota-encoded propionate production capacity was compared to fecal (F) and serum (G) propionate abundances. Linear mixed-effects models were used to calculate p values while adjusting for age as a fixed effect and HIV serostatus as a random effect. Spearman tests yielded rho values indicating direction of correlation. Axes are shown in square-root format. For all boxplots: center line, median; box limits, upper and lower quartiles; whiskers, 1.5× interquartile range.

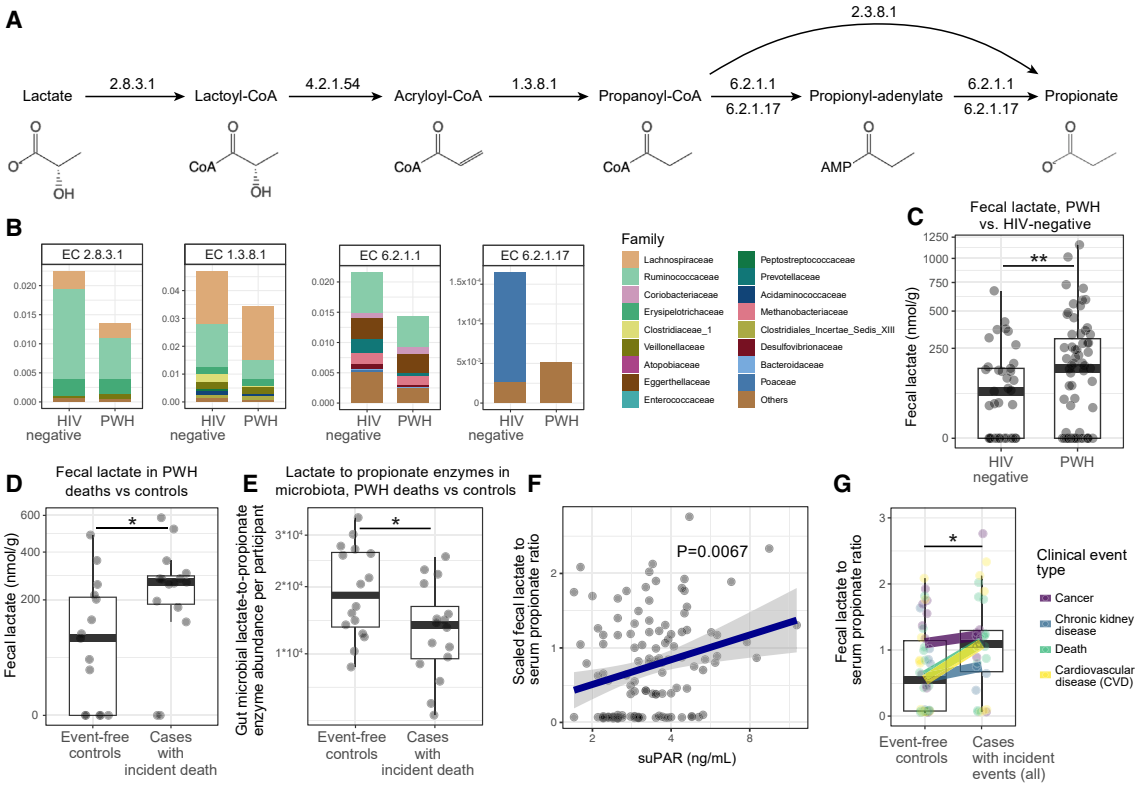

Figure 4. Conversion of fecal lactate to propionate by gut microbes is impaired in PWH and associates with inflammation and future death

(A) Lactate-to-propionate conversion pathway (excerpt of KEGG ko00640). EC numbers are shown above transformation lines.

(B) Relative abundances of all enzymes detected in dataset related to lactate-to-propionate conversion pathway shown in (A). Taxonomic origin of enzymes is shown.

(C) Fecal lactate concentrations are higher in PWH compared to people without HIV ($p = 0.0056$, conditional logistic regression; n = 58 PWH, n = 35 HIV-negative individuals).

(D) Fecal lactate concentrations are higher in PWH who died compared to PWH who did not ($p = 0.0267$, conditional logistic regression; n = 15 cases, n = 15 control subjects).

(E) Geometric means of relative abundances of gut microbial enzymes involved in lactate-to-propionate conversion (shown in panel A) are decreased in PWH who died (n = 16) compared to those who did not (n = 16 control subjects; $p = 0.0367$, conditional logistic regression).

(F) Scaled fecal lactate-to-serum propionate ratios correlate with serum suPAR ($p = 0.0067$ by linear mixed effects, n = 127 participants).

(G) Scaled fecal lactate-to-serum propionate ratios are elevated in incident PWH (n = 31) compared to event-free control subjects with HIV (n = 47 control subjects; $p = 0.0262$, conditional logistic regression). Axes are shown in square-root format. *$p < 0.05$, **$p < 0.01$. For all boxplots: center line, median; box limits, upper and lower quartiles; whiskers, 1.5× interquartile range.

inversely with prognostic inflammatory markers including suPAR. We found that HIV-associated shifts in gut microbiota composition were more pronounced in PWH who developed an incident comorbidity or died within the following 1–2 years as compared to PWH without an incident event. Systemic, but not fecal, concentrations of the SCFA propionate were significantly depleted in PWH, and the gut microbial pathway for conversion of lactate to propionate was especially depleted among PWH. Flux through the pathway converting lactate to propionate—as measured by fecal lactate-to-serum propionate ratios—correlated strongly with the key inflammatory prognostic marker suPAR, and this ratio was also elevated in PWH who experienced comorbidities or death compared to PWH who did not. We present a model in which the microbiome of ART-treated individuals with chronic HIV infection has diminished capacity to produce protective SCFA microbial metabolites, which permits inflammation and subsequent morbidity/mortality (model in Figure S6).

Several prior studies have investigated fecal SCFA content in PWH and found either minimal differences compared to people without HIV or mixed results.[28–31] Our results concordantly showed minimal differences in stool SCFA content between the PWH and HIV-negative subgroups matched for age, sex, sexual behavior, and alcohol consumption. Prior foundational studies have shown that uptake of colonic luminal SCFAs in humans occurs rapidly (within 30–60 min of infusion)[55,56] and with great efficiency (nearly 90% of SCFAs can be taken up from the lumen).[47] Therefore, the remaining SCFAs in stool are the balance of microbiota-mediated SCFA production minus host-mediated uptake. Using a previously published gene expression dataset,[46] we found that SCFAs themselves induce expression of SCFA transporters responsible for SCFA uptake in human

epithelial cells, suggesting that uptake is dependent on SCFA production and thus may not be constant between nor within individuals. Such dynamic regulation of uptake may exert significant variability upon readings of SCFAs that are left over in excreted feces. Since SCFAs induce expression of transporters responsible for uptake of SCFAs, a high luminal SCFA concentration can result in low final excreted SCFA concentrations due to increased uptake, producing a similar result in fecal concentration from a colon with low luminal SCFA concentration. Indeed, in line with such a model, fiber supplementation human intervention trials show a lack of robust increase in excreted fecal concentrations for both butyrate and propionate.[57] On the other hand, serum propionate correlated with abundance of enzymes involved in propionate production in the gut microbiota and did so more strongly than fecal propionate. We have thus presented evidence that measuring the absorbed pool of SCFAs—most notably propionate—in serum constitutes a valuable method for inferring total microbiota-mediated SCFA production that may more accurately represent bioavailable SCFAs than fecal SCFAs. Microbiota-encoded enzymes for butyrate production correlated neither with fecal nor serum butyrate, possibly because butyrate is the primary energy source for the colonic epithelium,[58] where it may be mostly metabolized and consumed. Because propionate and butyrate production enzymes in the gut microbiota correlated with each other in our dataset, it is possible that propionate—which is not the primary energy source for colonocytes and thus can more readily evade gut epithelial first-pass metabolism—in serum may be a useful biomarker of microbiota-mediated butyrate production as well. Isobutyrate was additionally diminished in serum of PWH. Depletion of isobutyrate in serum has been recently observed to correlate with diminished microbiota-mediated SCFA production in other human studies,[59] highlighting the concept that serum SCFAs may better reflect microbiota-mediated SCFA production. Such information may inform the design of trials for nutritional or microbiome-targeted therapies in humans to include measurements of circulating SCFAs.

Limitations of the study

Our study had several limitations that can be addressed in future studies. Our analyses were exploratory and aimed to discover associations between microbiome features, metabolites, and subsequent development of clinical events. Thus, multiple statistical comparisons were performed throughout the analysis, making observations prone to a potentially higher rate of false discovery. In addition, we relied on self-reported (validated) events at 2 year intervals, so it is possible that some cases of incident comorbidities were missed but were presenting later as cases of death or that some events were not reported, and people may have been inadvertently included as control subjects. Additionally, a few of the deaths were of people who already had a type of cancer prior to enrollment in the AGE_hIV cohort study. Cancer staging was not recorded, nor was positivity status for oncogenic viruses. Our cohort also did not have dietary intake information available from study participants. Prior studies that compared dietary intake between PWH and HIV-negative groups showed no significant differences,[37,60] suggesting that differences in diet alone are unlikely to drive the patterns we observed in microbiome-encoded SCFA production machinery and SCFA levels in PWH compared to HIV-negative individuals. Future studies aimed at understanding the basis of elevated comorbidity risk in PWH will likely benefit from detailed dietary intake data collection and examination of microbiome-mediated SCFA production. By selecting HIV-negative individuals that were stringently matched to the PWH in our subgroup analysis, comparability was achieved between the two subgroups at the expense of representativeness. For instance, the small number of females in the overall cohort hampered representation in our case-control study due to inability to find well-matched case and control subjects who are female, limiting the generalizability of our findings to females. In order to examine generalizability of family level taxonomic differences in our meta-analysis comparing PWH and HIV-negative groups across independent studies, we included available studies irrespective of confounder matching within each study. Notably, the results of our meta-analysis were consistent with a prior study in the AGE_hIV cohort in which known confounders were stringently matched.[14] Additionally, while our study in total comprised a large sample set (n = 138 participants), our sample sizes for specific comorbidities and mortality were considerably lower, and thus results may reflect idiosyncrasies of those specific participants. Validation of these observations in larger, well-powered prospective cohorts will more closely establish relationships between microbiome signatures and future clinical events. Despite our small sample size, our main findings support previous observations that suPAR and soluble C5 are likely important biomarkers of inflammation, signifying impending incident inflammatory comorbidity and mortality.[14,33,34]

Conclusions

By leveraging our observations regarding serum versus fecal SCFAs, we reported significant differences in serum levels of the SCFA propionate in PWH compared to people without HIV. Consistent with the well-described role of propionate in dampening innate and adaptive immune activation,[18–20] we found that serum propionate correlated with key markers of inflammation including suPAR. A difference in fecal lactate was noted in those who developed an incident comorbidity as well as in those who died. Lactate is a primary product of anaerobic glycolysis of fibers and other complex and simple carbohydrates. Such glycolysis reduces the electron acceptor nicotinamide adenine dinucleotide (NAD+), and gut microbes regenerate NAD+ by numerous chemical reactions including the conversion of lactate to propionate.[49,61,62] We found that PWH exhibit a depletion of microbial enzymatic machinery to facilitate this and other pathways for propionate production. This depletion was evident even when excluding all individuals (PWH and HIV-negative individuals) with receptive anal intercourse within the past 6 months, suggesting that recent receptive anal intercourse was not the driver of this depletion. This depletion of microbiome-encoded propionate production enzymes was associated with salient features of HIV-associated dysbiosis and lower alpha diversity, a phenomenon that is robustly reported across independent studies.[13–15] Fecal lactate can also be a byproduct of host metabolism during inflammation,[63] though regardless of its source, the inability to convert this abundant metabolite into

the host-protective propionate in PWH was linked with comorbidity onset in our study and plasma levels of suPAR, which is itself a strong predictor of clinical events and CVD in PWH.[34] This work highlights microbiota-mediated SCFA production, specifically that of propionate, as a target in the gut microbiota of treated, virally suppressed PWH for potentially modulating the risk of inflammation-related comorbidities and clinical disease progression.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2023.113336.

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AUTHOR CONTRIBUTIONS

Conceptualization, I.V.-C., I.S., and P.R.; methodology, I.V.-C., I.S., M.L.V., A.B., and P.R.; formal analysis, I.V.-C. and M.L.V.; investigation, I.V.-C., A.L., J.G., and M.L.V.; writing – original draft, I.V.-C., I.S., and M.L.V.; writing – review & editing, I.V.-C., I.S., M.L.V., and P.R.; visualization, I.V.-C.; funding acquisition, I.V.-C., I.S., and P.R.; resources, A.V., F.W.N.M.W., M.F.S.v.d.L., M.G., and N.A.K.; supervision, I.V.-C., I.S., and P.R.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human stool samples	University of Amsterdam	N/A
Human serum samples	University of Amsterdam	N/A
Critical commercial assays		
Human CD14 DuoSet ELISA DY383-05	R&D Systems	DY383
Human uPAR DuoSet ELISA	R&D Systems	DY807
Human IL-6 DuoSet ELISA	R&D Systems	DY206
Human Complement Component C5a DuoSet ELISA	R&D Systems	DY2037
Human FABP2/I-FABP DuoSet ELISA	R&D Systems	DY3078
KAPA library quantification Complete kit (Universal)	Roche	KK4824
MiSeq Reagent Kit v3 (600-cycle)	Illumina	MS-102-3003
Deposited data		
Raw 16S rRNA sequencing files (V4 hypervariable region)	Short Read Archive	PRJNA994573
Other		
Ampure XP SPRI reagent	Beckman Coulter	A63881
Phusion® High-Fidelity DNA Polymerase	New England BioLabs	M0530L
ZymoBIOMICS 96 DNA Kit	Zymo Research	D4309

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ivan Vujkovic-Cvijin (Ivan.Vujkovic-Cvijin@csmc.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Raw 16S rRNA sequencing data generated as part of this study have been deposited at the Short Read Archive (SRA) database under accession SRA: PRJNA994573. Data pertaining to quantification of short chain fatty acid are available in Table S6.
- This paper does not report original code
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human participants

The AGE$_h$IV Cohort Study is an ongoing observational cohort study of participants with HIV recruited from the outpatient HIV clinic of the Amsterdam University Medical Centers (UMC), location Academic Medical Center (AMC), and people without HIV recruited from either the sexual health clinic or the Amsterdam Cohort Studies on HIV/AIDS at the Public Health Service Amsterdam, the Netherlands. Details have been previously described.[7] In short, participants were 45 years or older at enrollment between 2010 and 2012. At baseline and every 2 years thereafter, participants undergo standardized screening for age-related comorbid conditions, and collection of blood, urine, and stool samples for cryopreservation. Study participants self-collected stool without preservative at their homes no more than 24 h prior to study visits and stored specimens in a refrigerator until being brought into the study location. Samples were stored at −80°C until processing for this study. Written informed consent was obtained from all participants

at enrollment and prior to onset of morbidity and mortality. The study was approved by the ethics committee of the Amsterdam UMC, location AMC and is registered at www.clinicaltrials.gov (NCT01466582). Microbiome and microbial metabolite investigations were exploratory analyses not pre-specified as primary or secondary outcomes. While study participants were followed longitudinally, single samples from each individual were analyzed for microbiome and metabolite levels. Detailed, de-identified characteristics of the human study participants investigated in this study are seen in Tables S1—S5.

METHOD DETAILS

Definitions of comorbidities and mortality

Self-reported and subsequently validated comorbidities included non-AIDS-defining cancers (confirmed by pathologist and excluding non-melanoma skin cancers); and cardiovascular diseases (CVD; diagnosed by specialist: myocardial infarction, angina pectoris, peripheral arterial disease, ischemic stroke, or transient ischemic attack).[64] Self-reported diagnoses were validated using hospital records for participants with HIV, and general practitioner (GP) records for people without HIV who provided consent to contact their GP. Non-validated diagnoses were excluded from the present analysis.

Incident chronic kidney disease (CKD) was defined as decreased kidney function (estimated glomerular filtration rate <60 mL/min/1.73m^2 using the Chronic Kidney Disease Epidemiology Collaboration equation) during two consecutive study visits, and normal kidney function ($\geq$60 mL/min/1.73m^2) at baseline.

For participants who died during follow-up, information on cause of death was obtained using hospital and GP records. For participants who were lost to follow-up or declined further participation in follow-up study visits mortality data were confirmed through the Municipal Personal Records Database.

Case-control study design for comparing participants with incident events of interest to event-free participants

We selected all AGE$_h$IV cohort participants who had at least one stool sample available during follow-up. For the selection of *cases*, we then selected those who were newly diagnosed with a non-AIDS-defining cancer, cerebrovascular accident (CVA), myocardial infarction, angina pectoris or CKD during follow-up, without such events known at baseline; or who died from any cause (i.e., incident event). For the current analysis, CVD events included CVA, myocardial infarction, or angina pectoris. We excluded cases in whom the diagnosis of the incident event was not validated and those without a stool sample prior to the date of the incident event.

Participants who were never diagnosed with any of the above listed events both at baseline and during study follow-up, and who were alive on October 1, 2018 were selected as potential *controls*.

We then aimed to match each case to two controls based on the following variables: HIV-status (HIV-positive or HIV-negative), sex at birth (male or female), age (closest to ±5 years at the time of stool sample collection), body mass index (BMI in kg/m^2), birth country ('the Netherlands', 'Surinam (Creole)', 'Netherlands Antilles or Aruba' or 'other'), sexual practice (men who have sex with men (MSM) or other), receptive anal intercourse in past six months (yes or no), smoking pack years (0, >0–6, >6–20, or >20 pack years), current smoking status (yes or no) and current alcohol consumption (yes or no). Matching was based on data obtained at the study visit directly prior to or at the date of stool sample collection. We performed coarsened exact matching (CEM) to obtain sets of matched cases and controls,[65] while choosing 3 to 4 equally-distributed cut offs to allow matching for the continuous variables age and BMI. We performed this matching procedure separately for each of the incident clinical events without replacement for individual controls. Given the distribution of matching variables for individuals who died, we were able to match each case of death to only one control. Matching for cases and controls was carried out using the 'cem' command in Stata (InterCooled v15.1, StataCorp, College Station, Texas). When considering all cases of interest to all controls, these two groups were balanced with respect to all matching variables, but cases were slightly older than controls (63.6 vs. 60.6 years, p = 0.044) within the *a priori* considered acceptable 5-year radius. Current CD4 and CD8 cell counts were not significantly different between cases and controls. In all individuals with HIV, cases and controls were likewise similar regarding years since HIV diagnosis and nadir CD4 cell count. A small number of samples failed quantification for some omics measurements (e.g., serum SCFA and fecal SCFA quantification) and were unable to be assessed. Total numbers of analyzed participants are denoted for each figure panel in figure legends. Of note, for five of the PWH who died of cancer, this cancer was already present at time of enrollment into the AGE$_h$IV cohort. In addition, six of those who died of cancer did not have their cancers reported as an incident event, likely because their cancer was diagnosed after the last study visit. Thus, these 11 were included only as cases of death but not as incident cancer cases. None of the study participants reported antibiotic use at the time of fecal collection. Of all participants, one (S0218, matched control for a CVD case) reported a single instance of intake of two tablets of azitromycin antibiotic (dose unknown) two months prior to fecal collection. Missing data (i.e., 16S sequencing, metabolite quantifications) for any participant was handled by removing the corresponding matched participant.

Selected subgroup for comparison by HIV-status

In order to compare the groups of PWH and HIV-negative participants, regardless of case/control status, we created a subset of those for whom strata of age, BMI, sex at birth, sexual behavior, and alcohol intake were represented in both groups. For these two subgroups, we first excluded participants for whom SCFA measurements and 16S sequencing profiles were not obtained. We then excluded participants for whom an individual stratum was absent in either PWH or HIV-negative participants: females were only present among HIV-negative participants and were thus excluded (n = 3); non-MSM were only present among PWH

and were excluded (n = 2); individuals with no current alcohol intake were only among PWH (n = 20 excluded). To balance ages between the two subgroups, the age range for both was restricted to 50–76 years, resulting in a further six individuals excluded. The final restricted subset totaled 66 and 36 individuals with and without HIV, respectively.

Soluble biomarker quantification

Soluble (s) CD14, sCD163, soluble urokinase plasminogen activator receptor (suPAR), IL-6, C5, kynurenine, tryptophan, and intestinal fatty acid binding protein (I-FABP) concentrations were determined in plasma samples stored at −80°C using enzyme-linked immunosorbent assay (ELISA) (DuoSet ELISAs; R&D Systems) in included participants.

DNA extraction and PCR amplification

DNA from stool samples was extracted using the ZymoBIOMICS 96 DNA Kit (Zymo Research). Batch effects were mitigated by ensuring a balanced representation on each DNA extraction plate of PWH and people without HIV, as well as including all matched pairs together on the same plate. Gut bacterial profiles of study participants were generated by broad-range amplification and sequence analysis of the V4 hypervariable region of the bacterial 16S rRNA gene. The V4 hypervariable region of the bacterial 16S rRNA gene was amplified using fusion primers with partial Illumina adaptors. The universal bacterial 515F forward primer (5′-GTGCCAGCMGCCGCGGTAA) and the 806R reverse primer (5′-GGACTACHVGGGTWTCTAAT) were used. PCR reactions were prepared in 100 μL volumes containing 20 μL of 5× Phusion High-Fidelity (HF) buffer, 2 μL of 10 mM dNTPs, 1 μL of each primer (50 μM), 0.5 μL of Phusion High-Fidelity DNA Polymerase, and 10 ng of template DNA. Master mixes were then split into triplicate reactions of 33 μL and amplified separately. V4 regions of the bacterial 16 S rRNA gene were amplified using PCR (98°C for 30 s, followed by 25 cycles at 98°C for 10 s, 57°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 5 min). The ZymoBIOMICS Microbial Community Standard was extracted for DNA along with the study samples and was used as a positive control. DNA elution buffer was used in separate wells as negative controls and all amplified insufficiently for sequencing.

Illumina MiSeq sequencing

Following amplification, triplicate samples were pooled and purified using Ampure XP Beads (1:1 ratio). Samples were then quantified using the KAPA qPCR-based Library Quantification kit and pooled at equimolar concentrations. Amplicons were paired-end sequenced (2 × 300 bp) on an Illumina MiSeq platform with a 600-cycle kit using standard protocols.

16S rRNA sequencing data processing and analysis

Raw fastq files were deposited to the Short Read Archive (SRA) using METAGENOTE.[66] Raw fastq files were processed using the dada2 algorithm (v1.3.3) after primer trimming and trimming amplicon lengths of the forward and reverse reads to 240 bp and 190 bp, respectively. Standard parameters were used (truncQ = 2, maxEE = 2). Taxonomy was assigned to amplicon sequence variants (ASVs) using the RDP training set 18. Fasta sequences for each ASV were processed through the md5sum hash algorithm (R library 'digest' v0.6.23) to create short, unique ASV identifiers. ASVs that were present in fewer than six samples were removed and ASV tables were rarefied to 18,000 reads for subsequent analyses. Alpha diversity metrics were calculated using R library 'vegan' v2.6-4. Imputation of gut microbiome function and estimation of gut microbial enzymes was performed using PICRUSt2[67] (v2.4.1) on rarefied ASV tables and outputs were Enzyme Commission (EC) tables for each study participant. Enzymes involved in propionate synthesis were identified as those within KEGG pathway ko00640 (Propanoate [propionic acid] metabolism), and those for butyrate metabolism were classified according to KEGG pathway ko00650 (Butanoate [butyric acid] metabolism). For calculating taxonomic contributions to the propionate metabolism pathway in people without HIV, relative abundance contributions to each enzyme were summed for each family-level taxonomic classification. Median family-level relative abundances across all people without HIV were calculated for each enzyme independently. Then, median values across all enzymes were subsequently calculated for each taxonomic family. Conditional logistic regression was performed using the 'survival' package in R (v3.5-5) and linear mixed effects tests were performed using the R packages 'lme4' v1.1-33 and 'lmerTest' v3.1-3.

HIV-associated dysbiosis index calculation

The HIV-Associated Dysbiosis Index ('HADI') was calculated as described previously.[14] 16S amplicon sequence variants that were enriched and depleted in the PWH compared to HIV-negative groups (having paired Wilcoxon $p < 0.05$ and log mean fold change >0 in the comparison of PWH vs. HIV-negative) were identified in a prior study[14] performed on a different subset of people within the same AGE_hIV cohort from which the present participants were drawn. Taxon identities are provided in Table S7. The abundance of these taxa in the present cohort of individuals were extracted from ASV tables generated as described above. The log ratio of geometric means of taxon abundances that were enriched in PWH over taxa that were depleted in PWH were calculated as follows:

$$DI = \log_{10}\left(\frac{\sqrt[n]{x_1 x_2 \ldots x_n}}{\sqrt[m]{y_1 y_2 \ldots y_m}}\right)$$

Where each x denotes read counts for taxa enriched in PWH and n is the total number of such taxa, while each y denotes read counts for taxa depleted in PWH and m is the total number of these taxa. Taxon read counts were used with an added pseudo-count of 1.

Gut microbiota meta-analysis in independent studies of people with HIV

Raw data were obtained from six independent studies[16,36–40] examining the microbiota of people with and without HIV that had publicly available 16S rRNA sequencing data (SRA accessions: SRP039076, SRP033311, PRJEB5185, PRJEB4335, ERP011842, SRP068240). Study participant numbers per study were as follows: SRP068240 (N = 78), SRP039076 (N = 36), SRP033311 (N = 63), ERP011842 (N = 98), PRJEB5185 (N = 24), PRJEB4335 (N = 31). One study (SRP033311) included 16S sequencing of both fecal and rectal biopsy samples, while the remaining studies were all fecal samples. Analyses were restricted to men who have sex with men and excluded men who have sex with women and individuals who reported intravenous drug use. Analyses were additionally restricted to chronic HIV-infected PWH, thereby excluding viremic controllers, elite controllers, and early infection study participants. Primer sequences were removed from all respective Fastq files using the 'fastq_filter' from USEARCH. Fastq files were subsequently processed using QIIME v1.9.1. Libraries were split according to metadata-provided barcodes and the maximum allowable quality score was set to 19. Open reference operational taxonomic unit picking was performed using the GreenGenes gg_13_8 database. Operational taxonomic unit tables were merged, and a minimum relative abundance filter threshold was set to 0.005%. Samples were rarefied to 2,000 reads to facilitate inclusion of the greatest number of studies and samples. Taxa were summarized to the family level using summarize_taxa_through_plots.py from QIIME according to assigned taxonomies. Linear mixed effects models were used to compare family abundances between the PWH and HIV-negative groups, with the study of origin and the sample type (biopsy/feces) as random effects. These data were used only to generate Figure 2A, and all remaining 16S-based analyses were performed on the data generated as part of this study of the AGEhIV cohort.

Transcriptomics of human epithelial cells exposed to SCFA

Data from Grouls et al.[46] were analyzed using normalized mRNA read counts obtained from the Gene Expression Omnibus database[68] (GSE200309), provided as reads per kilobase (RPK). In the cited study, iPSC-derived human intestinal epithelial cell layers were exposed to differing concentrations of SCFA and RNA-sequencing was performed.

Measurement of SCFA in serum

Human serum samples were spiked with stable labeled internal standards and homogenized and subjected to protein precipitation with an organic solvent. After centrifugation, an aliquot of the supernatant was derivatized. The reaction mixture was injected onto an Agilent 1290/AB Sciex QTrap 5500 LC MS/MS system equipped with a C18 reversed phase UHPLC column. The mass spectrometer was operated in negative mode using electrospray ionization (ESI). The peak area of the individual analyte product ions was measured against the peak area of the product ions of the corresponding internal standards. Quantitation was performed using a weighted linear least-squares regression analysis generated from fortified calibration standards prepared immediately prior to each run. LC-MS/MS raw data were collected using AB SCIEX software Analyst 1.6.3 and processed using SCIEX OS-MQ software v1.7.

Measurement of SCFA in feces

The method for the Nuclear Magnetic Resonance (NMR) analysis of fecal samples was adapted from the protocol developed by Kim et al.[69] for human fecal samples. The mass of each sample (about 700 mg) was carefully weighed prior to the sample preparation. To each sample tube 50 μL of 0.5 mm zirconium oxide beads (Next Advance, Inc.) ceramic beads and 500 μL of pH 7.4 potassium phosphate buffer (0.15 M) containing 0.2 mM NaN3 were added. Then, the tubes were subjected to bead beating for 30 s. The tubes were subsequently centrifuged at 16000 g at 4°C for 15 min. 400 μL of supernatant was transferred to new 1.5 mL Eppendorf tubes. These tubes were centrifuged at 16000 g at 4°C for 1 h. Then, 225 μL of supernatant was added to 25 μL of 100% D_2O containing 4 mM TSP-d4, and 6 mM dimethylsulfone. In the case of 5 samples sufficient supernatant could not be generated. For another 6 samples an additional 5-min centrifuge session was necessary to generate sufficient supernatant. A customized Gilson 215 liquid handler was used to transfer most of the samples to a 3.0 mm Bruker NMR tube rack. For some samples manual transfer was required.

1H NMR data were collected using a Bruker 600 MHz Avance *Neo*/IVDr spectrometer equipped with a 5 mm TCI cryogenic probe head and a z-gradient system. A Bruker SampleJet sample changer was used for sample insertion and removal. All experiments were recorded at 300 K. A standard sample 99.8% methanol-d4 was used for temperature calibration before each batch of measurements. One-dimensional (1D) 1H NMR spectra were recorded using the first increment of a NOESY pulse sequence with presaturation (γB1 = 50 Hz) during a relaxation delay of 4 s and a mixing time of 10 ms for efficient water suppression. Initial shimming was performed using the TopShim tool on a random mix of urine samples from the study, and subsequently the axial shims were optimized automatically before every measurement. Duration of 90° pulses were automatically calibrated for each individual sample using a homonuclear-gated mutation experiment on the locked and shimmed samples after automatic tuning and matching of the probe head. 16 scans of 65,536 points covering 12,335 Hz were recorded. The Free Induction Decay of the 1D experiment was zero-filled to 65,536 complex points prior to Fourier transformation. An exponential window function was applied with a line-broadening factor of 0.3 Hz. The spectra were automatically phase and baseline corrected and automatically referenced to an internal

standard (TSP = 0.0 ppm). Metabolites were quantified in a select number of spectra using the Chenomx NMR Suite version 8.6 and by fitting the remaining spectra automatically in the KIMBLE environment.[70] The areas were converted to concentrations using the dimethylsulfone internal standard.

QUANTIFICATION AND STATISTICAL ANALYSIS

Comparisons between cases with an incident event and controls for SCFA, soluble biomarkers, HADI, and Shannon alpha diversity were performed using conditional logistic regression. For linear analyses, fecal and serum SCFA concentrations were scaled (values divided by standard deviations across all values) and centered. To identify ASVs that were in differential abundance between cases and controls, conditional logistic regression tests were performed for all ASVs ('survival' v3.5-5 package in R). Comparisons of imputed enzyme abundances between PWH and HIV-negative participants were performed using linear models adjusting for age as a covariate (R package 'lme4'v1.1-31 and 'lmerTest' v3.1-3). Gene set enrichment analysis was performed by ranking enzymes by t values derived from linear mixed effects models testing differences in abundance between PWH and HIV-negative participants (R package 'fgsea' v3.16). Single values that encompass abundance of microbiota-encoded enzymes for propionate production were obtained by selecting all ECs within the ko00640 (Propanoate [propionic acid] metabolism) KEGG pathway. Enzymes with prevalence less than 50% in the dataset (<50% of samples have >0 reads) were excluded. Remaining 'zero' enzyme abundances were given a pseudo-count of 1, and a geometric mean was calculated across enzymes for each study participant. For linear models (conditional logistic regression and linear mixed effects models), outlier readings for serum and fecal SCFA were defined as readings that deviated more than three standard deviations from the scaled and centered mean and were removed. Comparisons of two continuous variables were performed using linear mixed effects using age, sex, and HIV serostatus as covariates if participants exhibited heterogeneity within these variables (e.g., had more than one value represented within a given set of individuals being compared). Accordingly, comparisons of serum and fecal propionate to microbiota-encoded propionate producing enzymes was performed using linear mixed effects models on all participants where data was available (including people with and without HIV) using geometric mean enzyme abundances as described above. Fecal lactate to serum propionate ratios were calculated by scaling (without centering) square-root transformed values for both data types. To facilitate ratios, zeroes in fecal lactate measurements were replaced with a pseudocount of '1' prior to transformation and scaling. Information regarding all statistical tests performed in this study can be found in figure legends. Significance was defined as alpha = 0.05.