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Specific prebiotics modulate gut microbiota and immune activation in HAART-naive HIV-infected adults: results of the "COPA" pilot randomized trial

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Intestinal mucosal immune system is an early target for human immunodeficiency virus type 1 (HIV-1) infection, resulting in CD4+ T-cell depletion, deterioration of gut lining, and fecal microbiota composition. We evaluated the effects of a prebiotic oligosaccharide mixture in highly active antiretroviral therapy (HAART)-naive HIV-1-infected adults. In a pilot double-blind, randomized, placebo-controlled study, 57 HAART-naive HIV-1-infected patients received a unique oligosaccharide mixture (15 or 30 g short chain galactooligosaccharides/long chain fructooligosaccharides/pectin hydrolysate-derived acidic oligosaccharides (scGOS/lcFOS/pAOS) daily) or a placebo for 12 weeks. Microbiota composition improved significantly with increased bifidobacteria, decreased *Clostridium coccoides/Eubacterium rectale* cluster, and decreased pathogenic *Clostridium lituseburense/Clostridium histolyticum* group levels upon prebiotic supplementation. In addition, a reduction of soluble CD14 (sCD14), activated CD4+/CD25+ T cells, and significantly increased natural killer (NK) cell activity when compared with control group were seen in the treatment group. The results of this pilot trial highly significantly show that dietary supplementation with a prebiotic oligosaccharide mixture results in improvement of the gut microbiota composition, reduction of sCD14, CD4+ T-cell activation (CD25), and improved NK cell activity in HAART-naive HIV-infected individuals.

INTRODUCTION

Human immunodeficiency virus (HIV) infection is characterized by progressive CD4+ T-cell depletion and immunodeficiency that paradoxically occur in the context of a chronic state of immune system activation. Early in HIV infection, a profound CD4+ T-cell depletion is found in the intestinal mucosa, resulting in deterioration of gut homeostasis.[1–3] The importance of gut-associated lymphoid tissue in the pathogenesis of HIV-1 infection has regained interest since Brenchley *et al.*[4] found that gut-derived bacterial components in the blood of infected subjects are increased upon disease progression. Both gut immune activation and inflammation, as well as decreased mucosal repair and regeneration, contribute to HIV-1-associated enteropathy, suggested to lead to increased bacterial compounds into the circulation.[5] These bacterial components (lipopolysaccharide (LPS), peptidoglycan, and bacterial DNA) may further stimulate the vicious circle of immune activation, which in turn promotes viral replication and disease progression. After antiretroviral therapy initiation, activation of CD4+ T cells still occurs and is related to less increase of CD4+ T cells over time and predicts more rapid disease progression.[6–8]

Recently, we showed that impairment of the gastrointestinal (GI) tract in HIV-1-infected adults is already present in the early phases of HIV-1 disease. The presence of opportunistic pathogens like *Pseudomonas aeruginosa* and *Candida albicans*

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was found to be 10-fold and 10,000-fold increased, respectively, compared with levels reported in a healthy population. In addition, lower levels of beneficial microbial groups were found, such as bifidobacteria and lactobacilli, compared with levels reported for the general population.[9] Both bifidobacteria and lactobacilli groups have a positive influence on mucosal immune function and gut health.[10] In addition, McKenna *et al.*[11] demonstrated altered gut microbiota composition in Simian immunodeficiency virus-infected macaques with colitis, strongly supporting the recent hypothesis that microbial alterations at GI-tract level are a key factor in the pathogenesis of chronic HIV infection.[9,12,13] The gut microbiota represents a crucial line of resistance to colonization by pathogens,[14] controls proliferation and differentiation of epithelial cells,[15] and modulates maturation and activity of both innate and adaptive immune system.[16,17] Impairment in microbiota composition can be addressed by using prebiotics. Prebiotics are nondigestible food ingredients, generally oligosaccharides, that modify intestinal microbiota balance by stimulating the growth of beneficial bacteria, such as bifidobacteria and lactobacilli.[18] Prebiotic oligosaccharides can act indirectly through microbiota-dependent mechanisms (i.e., rebalancing microbiota composition in the gut) and/or have a direct effect via activation or blockage of cellular receptors.[19] Prebiotic oligosaccharides can improve immune balance in infants, resulting in lower incidence of infections early in life, and correlate with improvement of intestinal microbiota with increased bifidobacteria levels.[20,21] Given the imbalanced microbiota composition and altered immune function already present in the earlier stages of HIV-1 infection, we investigated in a pilot study the possible microbial- and immune-modulating effects of dietary supplementation with a unique mixture of prebiotic oligosaccharides in highly active antiretroviral therapy (HAART)-naive HIV-1-infected adults.

RESULTS

Study population

Between June 2005 and May 2006, 57 HAART-naive HIV-1-positive adults were included and no differences between groups were observed with respect to baseline demographic and clinical characteristics (**Table 1**). Ten subjects did not complete the 12-week intervention period and were therefore excluded from per-protocol study group. Four subjects dropped out because of adverse events (15 g day^{-1}, $n=3$; 30 g day^{-1}, $n=1$), two subjects withdrew consent (15 g day^{-1}, $n=1$; 30 g day^{-1}, $n=1$), and four were lost to follow-up (15 g day^{-1}, $n=1$; 30 g day^{-1}, $n=1$; control, $n=2$).

Product tolerability

Both groups receiving the unique prebiotic mixture showed a nonsignificant increase in total GI score at week 4 of product intake compared with control group (1 and 2.5, respectively, for 15 and 30 g day^{-1} group). In the 30 g day^{-1} group, complaints significantly increased at week 12 compared with baseline. The main complaints were flatulence and abdominal distension. At 4 weeks after last product intake, scores had returned again to 0, similar to baseline (**Table 2**).

Product safety

Prebiotic supplementation did not result in any clinically relevant changes in biochemical safety parameters of liver function (plasma aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, and albumin) and renal function (plasma creatinine). Furthermore, no unexpected clinical adverse events (i.e., other than GI, product-related complaints, as discussed in the previous paragraph) or serious adverse events were observed in this HIV-1-infected treatment-naive study population.

Table 2 The composite GI symptom score (median (range)) for the ITT population (*n*=57)

	Controls (*n*=19)	scGOS/lcFOS/pAOS 15 g day^{-1} (*n*=19)	scGOS/lcFOS/pAOS 30 g day^{-1} (*n*=19)
Day 2/3	0 (0–2)	0 (0–3)	0 (0–8)
Week 4	0 (0–3)	1 (0–8)	2.5 (0–12)
Week 12	0 (0–6)	1 (0–6)	2 (0–6)[a]
Week 16	0 (0–2)	0 (0–3)	0 (0–5)[b]

Abbreviations: GI, gastrointestinal; ITT, intent-to-treat; lcFOS, long chain fructooligosaccharides; pAOS, pectin hydrolysate-derived acidic oligosaccharides; scGOS, short chain galactooligosaccharides.
[a]Significant difference (P=0.010) between 30 g day^{-1} dose and control group on within-subject change from week 12 to day 2/3.
[b]Significant difference (P=0.019) between 30 g day^{-1} dose and control group on within-subject change from week 16 to week 12.

Table 1 Patient demographic and clinical characteristics (mean±s.d.) at baseline (*n*=57)

	Total group (*n*=57)	Control (*n*=19)	scGOS/lcFOS/pAOS 15 g day^{-1} (*n*=19)	scGOS/lcFOS/pAOS 30 g day^{-1} (*n*=19)
Sex (M/F)	42/15	12/7	13/6	17/2
Age (years)	38.3±9.5	39.3±12.1	37.4±7.9	38.2±8.2
Height (cm)	172.0±8.2	170.8±8.1	172.0±9.1	173.1±7.8
Weight (kg)	71.3±14.9	68.3±16.9	71.7±16.1	73.8±11.4
BMI (kg m^{-2})	24.0±3.8	23.4±4.8	24.0±3.6	24.6±3.2
CD4$^+$ count (cells per μl)	520±161	502±149	536±173	519±166
HIV-1 RNA (copies per ml)	28.4±40.5×10^3	28.9±33.6×10^3	22.21±46.3×10^3	34.8±41.2×10^3

Abbreviations: BMI, body mass index; F, female; HIV-1, human immunodeficiency virus type 1; lcFOS, long chain fructooligosaccharides; M, male; pAOS, pectin hydrolysate-derived acidic oligosaccharides; scGOS, short chain galactooligosaccharides.

One serious adverse event, syphilis, occurred that was not related to the product.

Prebiotic effect of short chain galactooligosaccharides/long chain fructooligosaccharides/pectin hydrolysate-derived acidic oligosaccharides (scGOS/lcFOS/pAOS) on microbiota composition

After 12 weeks, a significant increase in bifidobacteria population (median (range)) was found compared with baseline in both 15 g day^{-1} (from 2.8% (0.0001–30.6) to 15.7% (1.2–36.4), $P=0.007$) and 30 g day^{-1} groups (from 3.6% (0.000001–22.3) to 18.9% (0.9–46.9), $P=0.01$), but not in the control group (from 1.5% (0.0–24.9) to 5.2% (0.3–19.0)) (**Figure 1a**). This change from baseline in both groups receiving prebiotic intervention was significantly higher ($P=0.009$ for 15 g day^{-1} group and $P=0.015$ for 30 g day^{-1} group) compared with the control group. In addition, a significant higher level of bifidobacteria population was detected at week 12 for groups receiving 15 g day^{-1} ($P=0.014$) and 30 g day^{-1} ($P=0.007$) dose, respectively, compared with the control group, suggesting improvement of bifidobacterial levels as depicted in **Figure 1a**.

In the higher-dose group of prebiotics (30 g day^{-1}), a significant decrease in *C. lituseburense*/*C. histolyticum* group, which includes pathogenic *Clostridium perfringens* and *Clostridium difficile* species (estimated marginal mean (95% confidence interval)), was found compared with baseline levels (from 0.016% (0.004–0.064) to 0.002% (0.001–0.007), $P=0.009$) (**Figure 1b**). In contrast, a significant increase (from 0.011% (0.003–0.041) to 0.044% (0.015–0.125), $P=0.030$) was observed in the control group. Moreover, at week 12, a significant lower level of *C. lituseburense*/*C. histolyticum* group was detected in both 15 and 30 g day^{-1} dose groups ($P=0.011$ and $P<0.001$, respectively) compared with the control group. In addition, a significant decrease from baseline of *Eubacterium rectale*/*Clostridium coccoides* cluster was found in both 15 g day^{-1} ($P=0.035$) and 30 g day^{-1} dose groups ($P=0.05$) (**Figure 1c**), whereas no change from baseline was seen in the control group. There were no changes found in other measured intestinal bacterial groups, including *P. aeruginosa*, *C. albicans*, *Bacteroides*/*Prevotella*, *lactobacilli*, *Escherichia coli*, and *Atopobium*.

Prebiotic effect of scGOS/lcFOS/pAOS on plasma LPS and soluble CD14 (sCD14) concentration

The levels of sCD14 were measured in plasma of all patients at baseline as well as after 4, 12, and 16 weeks of prebiotic intervention. The analysis of sCD14 levels in plasma showed that patients treated with 15 g day^{-1} had significantly lower levels after 4 and 12 weeks of treatment compared with placebo group (week 4: 9,952 pg ml^{-1} (s.d.±1,245) vs. 11,237 pg ml^{-1} (s.d.±1,342), $P=0.02$; week 12: 9,720 pg ml^{-1} (s.d.±1,442) vs. 11,302 pg ml^{-1} (s.d.±1,721), $P=0.02$) and significantly lower levels after 12 weeks compared with patients treated with 30 g day^{-1} (11,244 pg ml^{-1} (s.d.±1,997), $P=0.04$) (**Figure 2**).

LPS analysis did not show differences between treatment groups; however, patients treated with 15 g day^{-1} showed a significant decrease in LPS levels at week 16 compared with

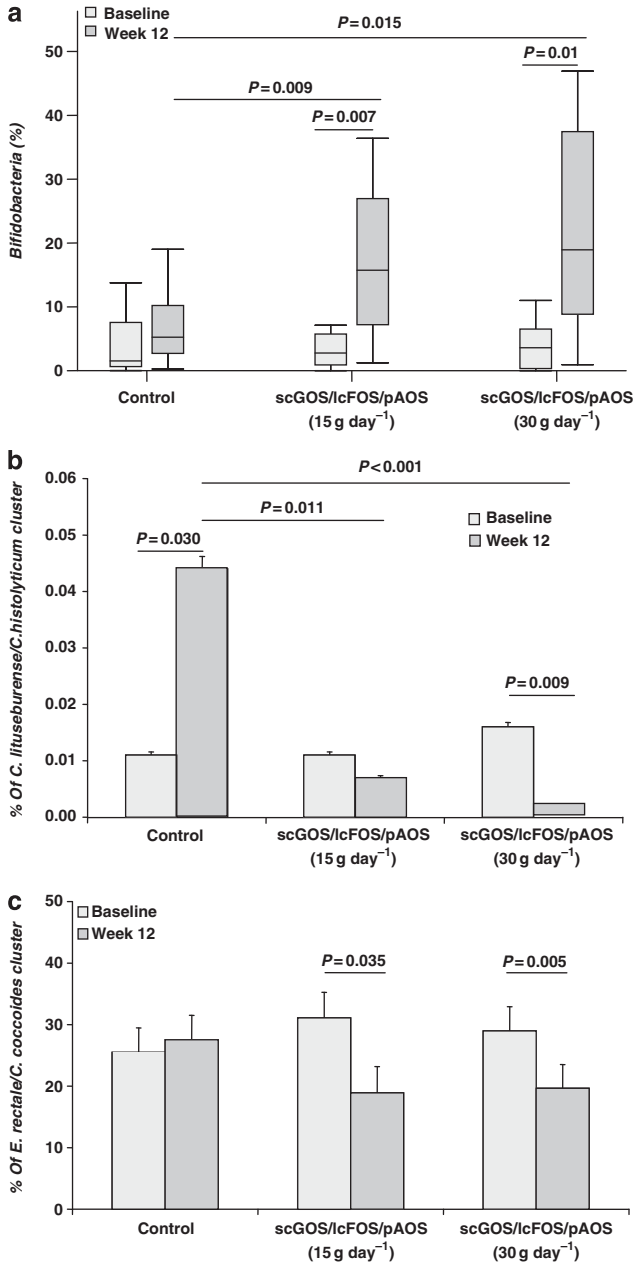

Figure 1 Improved gastrointestinal (GI) microbiota composition upon 12-week intake of short chain galactooligosaccharides/long chain fructooligosaccharides/pectin hydrolysate-derived acidic oligosaccharides (scGOS/lcFOS/pAOS). A 12-week prebiotic intervention with scGOS/lcFOS/pAOS in highly active antiretroviral therapy (HAART)-naive human immunodeficiency virus type 1 (HIV-1)-infected individuals was associated with (**a**) increased bifidobacterial levels expressed as median (range) percentage of total fecal bacteria, (**b**) reduced levels of the pathogenic *Clostridium histolyticum* cluster expressed as estimated marginal mean percentage (±s.e.) of the total fecal bacteria, and (**c**) reduced levels in the *Eubacterium rectale/Clostridium coccoides* cluster expressed as estimated marginal mean percentage (±s.e.) of total fecal bacteria. *P*-values indicate significant changes from either baseline within the groups as well as between groups (as tested with Mann–Whitney, analysis of variance (ANOVA)).

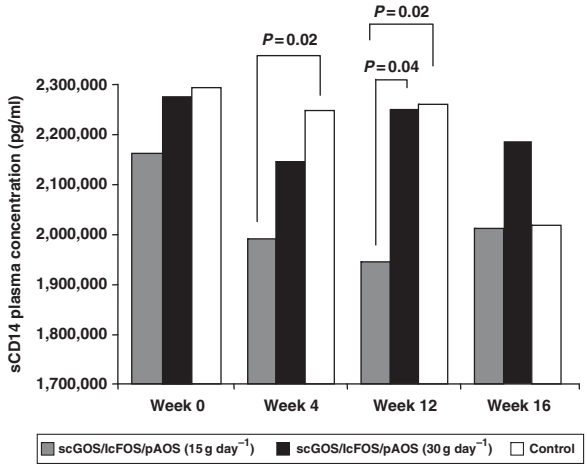

Figure 2 Effective reduction on plasma soluble CD14 (sCD14) concentration after administration of short chain galactooligosaccharides/long chain fructooligosaccharides/pectin hydrolysate-derived acidic oligosaccharides (scGOS/lcFOS/pAOS). Evaluation of sCD14 plasma levels at baseline and after 4, 12, and 16 weeks of prebiotic intervention. The analysis of sCD14 levels in plasma showed that patients treated with 15 g day^{-1} had significantly lower levels after 4 and 12 weeks of treatment compared with placebo group and significantly lower levels after 12 weeks compared with patients treated with 30 g day^{-1}.

baseline values (519.5 pg ml^{-1} (s.d.±59.2) vs. 588.2 pg ml^{-1} (s.d.±104.5), $P=0.04$).

scGOS/lcFOS/pAOS reduces immune activation of CD4$^+$ T cells

Although HIV-1 plasma viral load changes were found in groups receiving prebiotics (log viral load changed from 3.91±0.21 to 3.73±0.20 (15 g day^{-1}) and from 4.28±0.24 to 3.98±0.21 (30 g day^{-1}) (estimated marginal mean (±s.e.m.)) when compared with control (log viral load changed from 4.06±0.22 to 4.07±0.19 estimated marginal mean (±s.e.m.)), these were not statistically significant (**Table 3**). CD4$^+$ T-cell count did not change upon the short 12 weeks of dietary prebiotic supplementation, neither between groups nor within groups over time, as shown in detail in **Table 3**. Nevertheless, a dose-dependent reduction of activated CD4$^+$/CD25$^+$ T cells, expressed as percentage of total lymphocytes, was detected upon 12 weeks of prebiotic intervention (**Figure 3**). Subjects receiving the lower dose (15 g day^{-1}) showed a clear trend toward reduced levels of activated CD4$^+$/CD25$^+$ T cells (median (min–max)) after 12 weeks compared with baseline levels (from 0.36% (0.04–1.9) to 0.22% (0.02–1.25), $P=0.09$), whereas the higher-dose group (30 g day^{-1}) showed a statistically significant reduction compared with baseline (from 0.52% (0.01–7.21) to 0.27% (0.02–1.54), $P<0.01$). Compared with the control group, the group receiving 15 g day^{-1} dose showed statistically significant lower CD4$^+$ T-cell activation (CD25) at week 12 ($P<0.05$). The reduction in activated CD4$^+$/CD25$^+$ T-cell levels was not accompanied by a change from baseline in levels of activated CD8$^+$ T cells (as measured by percentage of CD8$^+$/CD38$^+$/CD45RO$^+$ T cells) nor by change from baseline in levels of FoxP3 (forkhead box P3)-expressing regulatory CD4$^+$/CD25^{++} T cells. Also, no significant changes from baseline were found in B7-H1 percentages on either monocytes or B cells (**Table 3**) or in cytokine profiles upon *ex vivo* re-stimulation with gag peptides or phytohemagglutinin (data not shown).

scGOS/lcFOS/pAOS improves natural killer (NK) cell activity

In addition to significant changes in the levels of activated CD4$^+$/CD25$^+$ T cells, NK cell activity significantly improved upon prebiotic intervention. The strongest effect was observed in the group receiving 15 g day^{-1} dose, with significant increases in all effector–target (E/T) ratios tested (12.5:1, 25:1, and 50:1) with respectively 4.25-fold ($P=0.002$) 3.63-fold ($P=0.001$), and 3.44-fold ($P<0.001$) higher levels compared with baseline (**Figure 3**). Similar effects, although not statistically significant for two of three ratios tested, were observed in the group receiving 30 g day^{-1} dose, in whom NK activity was increased from baseline 2.15-fold ($P=0.026$), 2.01-fold ($P=0.083$), and 1.93-fold ($P=0.107$) for E/T ratios 12.5:1, 25:1, and 50:1, respectively. No differences were found in control group compared with baseline. In addition, in the group receiving 15 g day^{-1} dose, NK cell activity at week 12 was significantly improved compared with subjects receiving control product at E/T ratios of 25:1 ($P=0.006$) and 50:1 ($P=0.003$).

DISCUSSION

Data herein suggest that dietary supplementation with a unique mixture of prebiotic oligosaccharides, consisting of scGOS/lcFOS/pAOS, may positively modulate gut microbiota composition, resulting in decreased sCD14 and LPS levels, CD4$^+$ T-cell activation (CD25), and increased activity of NK cells in HAART-naive HIV-infected adults.

The epithelial surface of the intestine is colonized by a high number of bacteria communities considered to be the first component of the defensive gut barrier. It represents a crucial line of resistance to colonization by pathogens, thereby decreasing the likelihood of bacterial translocation. Improved growth of bifidobacteria upon prebiotic feeding leads to a decrease in the pH and modulation of the short-chain fatty acids pattern that could contribute to the protection against pathogens. This study shows that supplementation with scGOS/lcFOS/pAOS mixture increased significantly the level of bifidobacteria in both intervention groups and reduced the levels of pathogenic clostridia-related species. Despite the reduced levels of pathogenic clostridia-related species, a full reduction of pathogenic load upon 12 weeks of intervention was not achieved, as levels of other pathogenic species like *P. aeruginosa* or *C. albicans* remained unchanged. In both prebiotic groups, a reduction in the level of *E. rectale/C. coccoides* cluster was observed. This bacterial cluster represents almost 75% of the *Firmicutes*, a major group of gut microbiota, including most of butyrate producers and has recently been implicated to play a role in human energy sequestration from diet.[16] Although specific relevance of alterations within this cluster to HIV-1 infection remains unclear, alterations found in this study are indicative for rebalancing intestinal microbiota

Table 3 Immune parameters; baseline and after 12 weeks of product intake (PP population)

EMM±s.e.m.	Control (*n*=17)	scGOS/lcFOS/pAOS 15 g day^{-1} (*n*=14)	scGOS/lcFOS/pAOS 30 g day^{-1} (*n*=16)	*P*-value[a]
CD4 count (cells per μl)				
Baseline	497±49.6	531±49.7	501±49.6	0.850
Week 12	548±66.8	478±71.5	520±74.9	0.745
Viral load (log_{10})				
Baseline	4.06±0.22	3.96±0.21	4.28±0.24	0.564
Week 12	4.07±0.19	3.73±0.20	3.98±0.21	0.422
CD4 (%)				
Baseline	23.2±2.3	24.6±2.4	24.6±2.5	0.862
Week 12	24.7±2.3	23.4±2.2	25.2±2.3	0.821
CD8 (%)				
Baseline	48.2±3.1	49.5±3.3	48.5±3.3	0.953
Week 12	48.8±3.3	50.5±3.5	48.8±3.6	0.909

Median (range)	Control	scGOS/lcFOS/ pAOS 15 g day^{-1}	*P*-value[b]	scGOS/lcFOS/ pAOS 30 g day^{-1}	*P*-value[b]
%CD8$^+$/CD38/CD45RO$^+$					
Baseline	13.2 (5.2–19.1)	7.3 (3.4–15.7)	0.009	8.4 (3.0–17.7)	0.073
Week 12	8.6 (0.7–42.1)	8.6 (1.7–16.0)	0.940	6.8 (0.2–38.8)	0.335
%CD4$^+$/CD25$^+$/FoxP3$^+$					
Baseline	1.12 (0.38–4.35)	0.85 (0.15–6.08)	0.158	0.74 (0.22–2.69)	0.408
Week 12	0.73 (0.30–8.88)	1.03 (0.11–2.43)	0.118	0.78 (0.20–4.69)	0.910
%CD14$^+$/B7-H1$^+$					
Baseline	14.9 (0.23–31.7)	15.6 (0.70–45.9)	0.565	16.5 (0.11–62.6)	0.169
Week 12	16.4 (0.00–25.0)	18.5 (1.32–41.0)	0.615	14.6 (0.65–45.4)	0.487
%CD19$^+$/B7-H1$^+$					
Baseline	2.95 (0.12–17.5)	4.42 (0.87–9.38)	0.688	3.50 (0.69–11.3)	0.766
Week 12	3.01 (0.00–7.33)	5.64 (0.36–11.8)	0.054	3.37 (0.67–8.63)	0.958

Abbreviations: EMM, estimated marginal mean; lcFOS, long chain fructooligosaccharides; pAOS, pectin hydrolysate-derived acidic oligosaccharides; PP, per protocol; scGOS, short chain galactooligosaccharides.
[a]Tested with ANOVA (α=0.05), testing for overall differences between the three intervention groups.
[b]Mann–Whitney, comparison with control group (multiple comparisons→α=0.10/3=0.033).

in treatment-naive HIV-1 infected upon supplementation with the specific prebiotic mixture. This reassess is also confirmed by the significant decrease of sCD14 plasma levels in the patients receiving 15 g day^{-1} compared with placebo group and by the reduction of LPS plasma levels at week 16 in patients treated with 15 g day^{-1} compared with baseline values.

Although in this study a healthy control group was not included, we recently reported *Bifidobacterium* levels to be lower in HAART-naive HIV-1-infected adults than those reported in the general population.[9] Modulating the GI tract toward a bifidogenic microbiota has long been regarded as a beneficial health effect for the host. Bifidobacteria supplementation has been associated with lower bacterial translocation, leading to a decrease in the inflammatory cascade activation in several models of bacterial translocation.[22] Similarly, other studies have demonstrated that modulation of the gut microbiota via prebiotic or probiotic ingestion may improve or prevent disruption of intestinal permeability in animal models and humans.[23] Furthermore, increased levels of fecal bifidobacteria have been shown to reduce intestinal LPS in murine

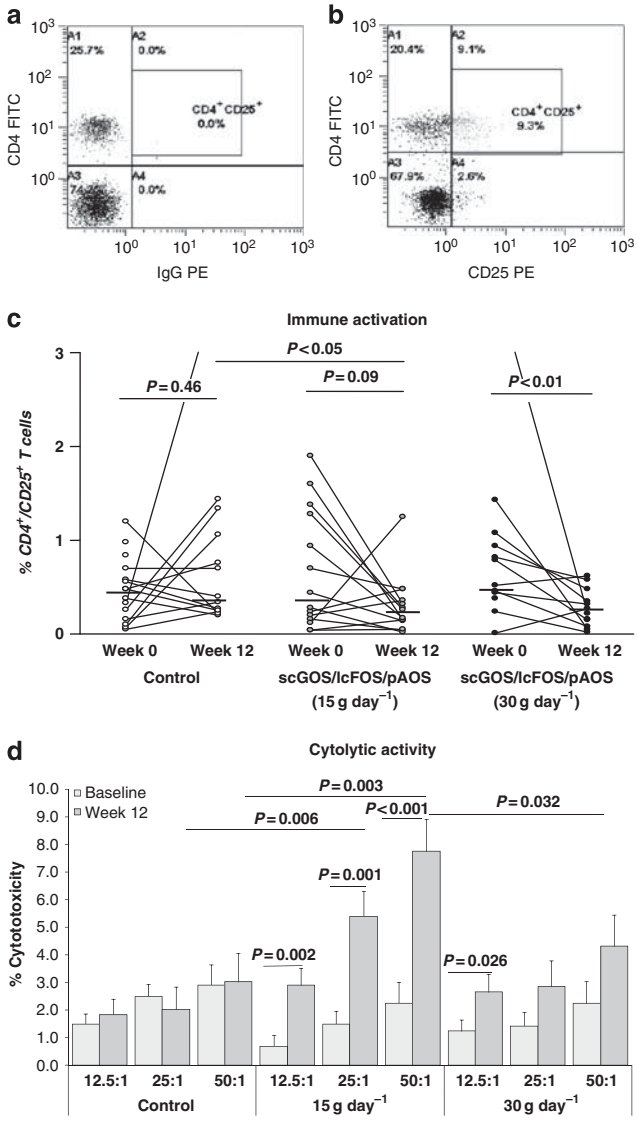

Figure 3 Beneficial immune changes because of intake of short chain galactooligosaccharides/long chain fructooligosaccharides/pectin hydrolysate-derived acidic oligosaccharides (scGOS/lcFOS/pAOS). Flow cytometry measurement of CD25 expression on $CD4^+$ T cells on lymphocytes first gated on CD45 and CD3. In (**a**) cells are visualized labeled with isotype control, whereas (**b**) represents the CD25 labeling. The 12-week scGOS/LcFOS/pAOS intake was associated with (**c**) reduced $CD4^+$ T-cell activation and (**d**) improved natural killer (NK) cell cytotoxicity. Individual percentages of $CD4^+/CD25^+$ T cells are shown as dots, with median values displayed per group as lines. The *P*-values indicate statistical significant changes from baseline as tested with Mann–Whitney ($\alpha = 0.033$ for three multiple comparisons). Percentage cytotoxicity is expressed as estimated marginal means (±s.e.) of target cell lysis at indicated effector–target (E/T) ratios. The *P*-values indicate significant changes from baseline within the groups as well as between groups (analysis of variance (ANOVA)).

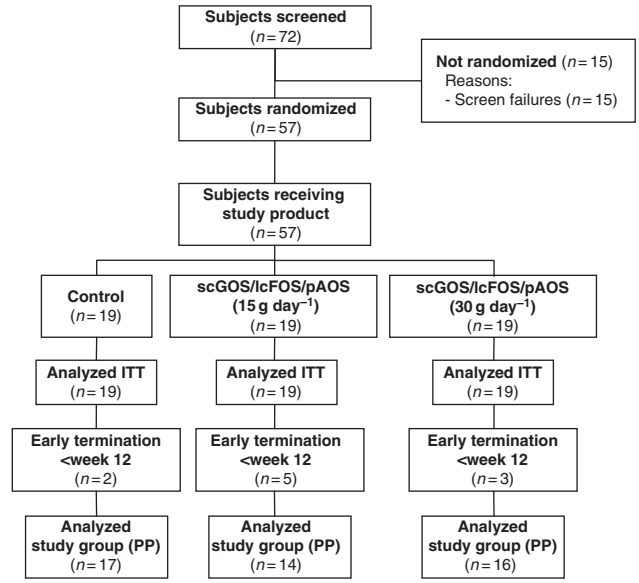

Figure 4 Overview of patient inclusion. Patients were randomized based on study in and exclusion criteria and evenly divided over the three arms. Patients who did not complete the 12-week supplementation period were excluded from the per-protocol (PP) study group and analyzed in the intent-to-treat (ITT) study group. lcFOS, long chain fructooligosaccharides; pAOS, pectin hydrolysate-derived acidic oligosaccharides; scGOS, short chain galactooligosaccharides.

models and to improve the mucosal barrier function.[24] Thus, particular bacterial species from the intestinal microbiota, including bifidobacteria, may well possess immunomodulatory properties during HIV-1 infection, although that study population was different compared with the HAART-naive subjects in our study.[25–28] In addition, probiotic supplementation has been indicated to possibly influence CD4 count.[29]

As it is well recognized that translocation of luminal bacteria and toxins is linked namely to disruption of the normal balance in the gut microbiota, impaired immune function and gut barrier function,[30] it is postulated that the clinical benefits from consumption of prebiotics are obtained through their effect on the colonic microbiota or directly through the immune system. In addition to the observed intestinal microbiota changes in our study, a clear dose-dependent inhibition of $CD4^+$ T-cell activation, although only measured by CD25 expression, was demonstrated. Although no statistically significant effect of the intervention was found on $CD4^+$ T-cell levels in HIV-1-infected adults and only a slight reduction (0.2–0.3 log) in viral load was observed. T-cell activation in our study was measured by the expression of CD38, memory $CD45RO^+CD8^+$ T cells, and CD25 on $CD8^+$ and $CD4^+$ T cells, respectively. In contrast to the reduced $CD4^+$ T-cell activation, the CD38 expression on $CD8^+$ T cells was not altered, although these data may have been distorted because of high variation and differences in activation of CD38 by memory ($CD45RO^+$) $CD8^+$ T cells, a measure of pathologic immune activation in HIV-1 infection associated with disease progression (**Table 3**).

In light of the critical role of regulatory T cells in the regulation of immune cells, and suggested role in immunopathology during chronic HIV-1 infection also, the number of regulatory T cells was measured in our study. A rapid disease progression seems to be associated with the expansion of regulatory T cells

in the course of HIV-1 infection. In addition, Cao *et al.*[31] recently suggested that $CD4^+$, but not $CD8^+$, T-cell activation was correlated with the proportion of regulatory T cells in HIV-1-infected individuals.[31] The present results did not indicate any modification in the number of $CD4^+/CD25^+/Foxp3^+$ regulatory T cells as result of 12 weeks of prebiotic intake.

One of the notable findings from our preliminary study is that upon prebiotic supplementation, a significant improvement in NK cell cytotoxicity was found, with the most pronounced effect in 15 g day^{-1} dose group. This suggests that for NK cell improvement the effect can already be reached using 15 g day^{-1} and there is no additive value on this marker to use the 30 g day^{-1} dose. In HIV-1 infection in humans, an inverse association exists between viral load/replication during chronic infection and both NK cell frequency and function.[32–35] Our finding might be explained by reduced levels of translocated bacterial components from the gut upon re-establishment of the gut microbial ecosystem, as chronic exposure to LPS, which is known to be related to reduced NK cell cytotoxicity.[36] Furthermore, programmed cell death-1 (PD-1)/PD-1L pathway, was previously found to be associated with NK and T-cell dysfunction, in that upregulation of PD-1 expression in rapid progressors is associated with a reduced cytolytic activity, i.e., perforin and interferon-γ production.[37] However, no significant changes were observed on either B7-H1 expression on monocytes nor on $CD8^+$ T-cell activation upon 12 weeks of product intake. Taken together, the mechanism explaining the significant improvement in NK cell cytotoxicity as well as the reduction of HIV-1-induced immune activation by prebiotics remains to be elucidated.

To confirm acceptance of prebiotic supplementation in HIV-1-infected adults, tolerability and safety of scGOS/lcFOS/pAOS mixture upon prolonged intake (12 weeks) was determined. In general, prebiotics might lead to gut symptoms including flatulence and abdominal distension. Therefore, initial increase observed in total GI score at week 4 was anticipated. The increase however was nonsignificant, and in general only "mild" scores in GI symptoms were recorded from subjects. Furthermore, a decrease in GI symptom incidence at the end of intervention was found compared with the incidence at week 4, which may well be explained by adaptation of microbiota composition and therefore improved/adapted fermentation of prebiotics. Apart from the observed GI symptoms, no other safety issues were identified (as demonstrated by liver and renal safety markers) with supplementation of this unique prebiotic mixture in HAART-naive HIV-1-infected adults. Therefore, the tested prebiotic mix can be regarded as tolerable and safe for this particular target population.

In conclusion, our study, although preliminary, suggests that in nonsymptomatic HAART-naive HIV-1-infected adults, gut microbiota can at least partially be restored by a unique prebiotic mixture consisting of scGOS/lcFOS/pAOS, with stimulation of bifidobacteria growth and reduction in fecal pathogenic load. In addition, we demonstrated a significant reduction in sCD14 and LPS levels, $CD4^+$ T-cell activation (CD25), and improved NK cell cytolytic activity in these HIV-1-infected adults after prebiotic supplementation. The mechanisms responsible for these observations remain to be elucidated as well as persistence of these effects upon prolonged supplementation. Although prebiotic dietary fibers have been extensively investigated for their therapeutic effects in other settings, to our knowledge this is the first study demonstrating clear health beneficial effects of prebiotics in HIV-1 infection. Despite that statistically significant differences were observed, the data presented herein came from a small sample size patient cohort. It will therefore be important to validate these results in a larger cohort of HIV-infected individuals.

METHODS

Study design

In a double-blind, randomized, placebo-controlled, pilot study (COPA trial: *C*linical trial with *O*ligosaccharides *P*owder for *A*pplication in HIV-1 infection), 57 nonsymptomatic HAART-naive HIV-1-positive adults were recruited in three Italian centers. Subjects were randomized in three groups, receiving three sachets of powder (16 g per sachet) daily. In two of these groups, the powder contained three different prebiotic oligosaccharide materials, i.e., scGOS (Borculo Domo, Zwolle, The Netherlands; 45% scGOS), lcFOS (Orafti, Wijchen, The Netherlands; 100% lcFOS), and pAOS (Sudzucker, Mannheim, Germany; 85% galacturonic acid). The prebiotics were mixed in a ratio of 9:1:10 based on prebiotic purity, with one group receiving 15 g day^{-1} and the other 30 g day^{-1} of these prebiotics. The control group received solely digestible sugar maltodextrin. In order to get accustomed to the oligosaccharides, which could lead to GI effects like flatulence, subjects gradually increased product intake with the complete daily dose from the third week onward. Total study duration was 16 weeks, consisting of 12 weeks of intervention and 4 weeks of follow-up. Product tolerability and safety were assessed on the intent-to-treat study group. In order to analyze both immune and microbiota responses related to the intervention, these parameters were analyzed from those subjects who completed the 12-week supplementation period as depicted in more detail in **Figure 4**. All analysis and laboratory staff members were blinded during the entire duration of the study. The study was approved by the institutional review board of the "Luigi Sacco" Hospital, Milano, Italy, by the institutional review board of the Busto Arsizio Hospital, Busto Arsizio, Italy, and by the institutional review board of the Spedali Civili di Brescia Hospital, Brescia, Italy. The study has been conducted in compliance with "Declaration of Helsinki" principles and with local Italian laws and regulations. Written informed consent was obtained from all subjects before study participation.

Product tolerability and safety

GI tolerability was assessed on days 2 or 3 from baseline and at weeks 4, 12, and 16 by a 4-point scale recall questionnaire based on Van Aerde *et al.*[38] Seven GI symptoms (nausea, burping, abdominal distention, flatulence, diarrhea, constipation, and urgent need for defecation) were each scored by the patients on "0" (no complaint) to "3" (severe complaint) scale. Total GI score was calculated as the sum of all scores. For product safety, liver function (plasma aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, and albumin) and renal function (plasma creatinine) were assessed at baseline and week 12.

Gut microbiota composition

Fecal sample collection and preparation. Stool samples, collected at baseline and after 12 weeks, were immediately stored at −20 °C until further processing. Frozen samples were thawed on ice water and 0.5 g was fixed with paraformaldehyde as described previously.[39] For DNA extraction, 0.2 g fecal sample was resuspended in 0.05 M phosphate-buffered saline. DNA was isolated using bead beating method as described previously.[39]

Real-time PCR. Quantification of total bifidobacteria, lactobacilli, and *P. aeruginosa* was performed with real-time PCR using duplex 5′-nuclease assay. Measurement of bifidobacteria and lactobacilli was performed as previously described.[39,40] *P. aeruginosa* was determined using the method described by Pirnay *et al.*[41] with some minor modifications; fluorescent labels were changed to 6FAM and TAMRA. Fluorescence signal was measured in annealing phase on ABI 7900HT Fast (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Total bacterial load was determined as described by Nadkarni *et al.*[42] Relative percentage of *P. aeruginosa* was subsequently calculated using comparative Ct method according to Liu *et al.*[43]

Fluorescent *in situ* hybridization. Oligonucleotide probes used in this study were: (i) Chis150 to detect *C. histolyticum* group,[44] (ii) Clit135 for *C. lituseburense* group,[44] (iii) Bac303 for *Bacteroides/Prevotella* group,[45] (iv) Erec482 for members of *E. rectale/C. coccoides* cluster,[44] (v) Eco1531 for *E. coli* and related species,[46] (vi) Caal for *C. albicans*,[47] and (vii) Ato291 for *Atopobium* cluster.[48] These probes were covalently linked at their 5′-end either to fluorescein isothiocyanate (FITC) or Cy3. Fixed fecal samples were hybridized with probes as described previously.[49] Fluorescent cells were then counted automatically[50] with a Leica DMRXA epifluorescence microscope (Leica, Wetzlar, Germany). For each analysis, 25 microscopic fields were counted and the target bacterial groups were recorded as a percentage of total bacterial cell count.[51]

Immunological markers

Blood collection, $CD4^+$ T-cell counts, and viral load measurements. Whole blood was collected by venipucture in Vacutainer tubes containing EDTA (BD Biosciences, Erembodegem, Belgium). $CD4^+$ T-cell counts were determined by flow cytometry. Plasma HIV-1 RNA levels were quantified by nucleic acid signal-amplification assay with detection limit of 50 copies per ml. Peripheral blood mononuclear cells (PBMCs) were separated on lymphocyte separation medium (Organon Teknika, Dublin, Ireland), and washed twice in phosphate-buffered saline. Freshly isolated PBMCs were kept at room temperature and used within 12 h. Additionally, PBMCs were also frozen in dimethyl sulfoxide and stored in liquid nitrogen until further use (see also FoxP3 staining and NK cell activity).

Immunophenotypic analyses. Lymphocyte subsets were evaluated by flow cytometric analysis, using 50 µl of EDTA peripheral blood incubated for 30 min at 4 °C with fluorochrome-labeled monoclonal antibodies (CD4 R-PE-Cyanine 5 Tandem–PE-Cy5-; CD3 PE-Cy5; CD8 PE-Cy5; CD14 PE-Cy5; CD19 PE-Cy5; CD25 PE; CD38 PE; CD8 FITC; CD45RO FITC) (Caltag Laboratories, Burlingame, CA). After incubation, erythrocyte lysis and fixation was performed using Immuno-Prep EPICS kit (Coulter Electronics, Milano, Italy) and Q-prep Work Station (Coulter Electronics). For indirect immunofluorescence staining, freshly isolated PBMCs were incubated with mouse-anti-human B7-H1 antibody (2.5 µg ml^{-1}) a gift of Dr L. Chen (Johns Hopkins University School of Medicine, Baltimore, MD). After 30 min at 4 °C, cells were washed and incubated for 30 min at 4 °C with rat-anti-mouse IgG (H + L) Ab-FITC (1 µg ml^{-1}) or with a mouse-anti-hamster IgG-PE (1 µg ml^{-1}) (Caltag Laboratories).

Cytometric analysis. Cytometric analyses were performed using an EPICS XL flow cytometer (Beckman Coulter, Brea, CA). For each analysis, 20,000 events were acquired and gated on CD4 (or CD8, CD14, CD19) expression and side scatter properties. For FoxP3 intracellular staining, cryopreserved PBMCs were thawed and washed with phosphate-buffered saline containing 2% bovine serum albumin. Subsequently, 1×10^6 cells were stained according to the manufacturer's protocol (eBioscience, San Diego, CA). Acquisition of data was performed on the same day as staining.

Plasma LPS and sCD14 concentration. LPS and sCD14 concentration was measured on plasma samples using LAL Chromogenic Endopoint Assay (Hycult Biotechnology, Uden, The Netherlands) and sCD14 ELISA kit (R&D Systems, Minneapolis, MN; Bender MedSystems, Vienna, Austria), respectively. All the analyses were conducted following the manufacturer's instructions. Plasma concentration of each protein was calculated relatively to standard curve.

NK cell cytotoxicity. Cytotoxic activity of NK cells against target cell line (K562) was measured by flow cytometry using NKTEST-kit in accordance with the manufacturer's instructions (ORPEGEN Pharma, Heidelberg, Germany). In brief, K562-target cells were labeled with lipophilic green fluorescent membrane dye discriminating effector and target cells. Cryopreserved PBMCs were thawed, washed, and subsequently incubated at ET ratios of 50:1, 25:1, and 12.5:1. After 4 h of incubation, killed target cells are identified by DNA stain. Percentage of target cells killed by effector NK was determined using FC 500 (Beckman Coulter).

Statistical analyses

Comparisons are made between results obtained at week 12 compared with baseline for all three groups. Also, change from baseline was compared between control group and the two treatment groups. The statistical analyses used for these comparisons are analysis of variance and Dunnett's test for multiple comparisons using SPSS (SPSS, Chicago, IL) version 12.1 or higher. For two comparisons, P-values of <0.05 were considered to be statistically significant, and for three comparisons, α of 0.10 was used. In case data were not normally distributed, log transformations were performed to obtain normal distributions and values are reported as estimated marginal mean with s.e.m. When transforming the data did not result in normal distribution of the parameter, nonparametrical testing (Mann–Whitney) was performed and values are reported as median with range.

DISCLOSURE

Belinda van't Land, Kaouther Ben Amor, Jan Knol, Johan Garssen, Jacqueline van Schaik, and Aldwin Vriesema are employees of Nutricia Advanced Medical Nutrition, Danone Research–Centre for Specialised Nutrition, Wageningen, The Netherlands. Kadija Benlhassan-Chahour and Dorothy Bray are employees of ImmunoClin Paris, France. There has been no personal financial interest of any of these people with respect to planning, executing, and reporting the study or any planned event afterwards.

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AUTHOR CONTRIBUTIONS

A.G., B.V.L., J.V.S., J.K., D.B., A.V., J.G., and M.C. conceived and designed the experiments; A.G., G.R., G.W., C.T., T.Q., C.T., and A.B. performed the experiments; A.G., G.R., B.V.L., K.B.A., J.V.S., J.K., D.B., A.V., J.W., J.G., and M.C. analyzed the data; K.B.A., C.T., T.Q., C.T., A.B., K.B.C., D.T., and J.W. contributed reagents/materials/analysis tools; A.G., B.V.L., and M.C. wrote the paper.

REGISTRATION NUMBER AND NAME OF TRIAL REGISTER

This study was registered in the Clinical Trial Database under ISRCTN30730587 and title Tolerance and immunological response in human immunodeficiency virus (HIV) seropositive individuals after NR100063 supplementation.

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