Title

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Permalink

https://escholarship.org/uc/item/4vs4802r

Journal

Open Forum Infectious Diseases, 6(11)

ISSN

2328-8957

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Publication Date

2019-11-01

DOI

10.1093/ofid/ofz434

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Peer reviewed

Changes in the Fungal Marker β-D-Glucan After Antiretroviral Therapy and Association With Adiposity

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Background. Bacterial translocation in HIV is associated with inflammation and metabolic complications; few data exist on the role of fungal translocation.

Methods. A5260s was a substudy of A5257, a prospective open label randomized trial in which treatment-naïve people with HIV (PWH) were randomized to tenofovir-emtricitabine (TDF/FTC) plus atazanavir-ritonavir (ATV/r), darunavir-ritonavir (DRV/r), or raltegravir (RAL) over 96 weeks. Baseline was assessed, and changes in β-D-glucan (BDG) were assessed at weeks 4, 24, and 96. Wilcoxon rank-sum tests were used to compare distribution shifts in the changes from baseline between treatment arms and linear regression models to assess associations between BDG and measures of inflammation, body composition, and insulin resistance.

Results. Two hundred thirty-one participants were randomized; 90% were male, the median age was 36 years, HIV-1 RNA was 4.56 log10c/mL, and CD4 cell count was 338 cells/mm^3. There was an overall increase in BDG over 96 weeks (1.57 mean fold-change; 95% confidence interval, 1.39 to 1.77) with no differences between arms. Twofold higher BDG levels at week 96 were associated with increases in trunk fat (8%) and total fat (7%) over 96 weeks ($P \leq .035$). At week 4, BDG correlated with I-FABP, a marker of enterocyte damage, and zonulin, a marker of intestinal permeability ($r = .19$–$.20$; $P < .01$).

Conclusions. In treatment-naïve participants initiating antiretroviral therapy (ART) with TDF/FTC and either RAL or ATV/r, DRV/r, BDG, a marker of fungal translocation, increased similarly in all arms over 96 weeks. This may represent continued intestinal damage during ART and resulting fungal translocation. Higher BDG was associated with larger fat gains on ART.

Keywords. fat; fungal translocation; gut integrity; HIV; inflammation.

Metabolic complications remain an important clinical issue facing people with HIV (PWH). Changes in weight and central fat gain continue to be reported with contemporary antiretroviral therapy (ART) in PWH. With older ART, fat abnormalities were dominated by lipoatrophy [1–3]. However, lipohypertrophy continues to be prominent and substantial consequence to the success of ART. These fat alterations not only cause major cosmetic concerns, but are also associated with heightened inflammation [4] and increased diabetes and CVD risk [5]. Initially thought to be linked to the use of protease inhibitors (PIs), we previously reported that participants initiating therapy with integrase inhibitors (INSTIs) experienced the same extent of visceral adipose tissue (VAT) gains when compared with participants initiating PI-based therapies [6]. Although the relationships between inflammation and

these fat alterations are likely bidirectional, the triggering factor linking inflammation to these metabolic alterations has yet to be determined.

Inflammatory states, such as HIV, are known to induce disruption in intestinal integrity [7, 8], which can cause damage to the gut and increase microbial translocation. This excess microbial translocation can lead to downstream transcription and activation of pro-inflammatory mediators [9]. Although fungi are an integral part of the human microbiota, their contribution to systemic inflammation remains largely unknown. Markers such as β-D-glucan (BDG), a polysaccharide cell wall component of most fungal species, is known to be highly immunogenic, stimulating macrophages, neutrophils, and T cells and leading to release of pro-inflammatory cytokines such as interleukin (IL)-8 and tumor necrosis factor α [10, 11]. We have previously observed that markers of fungal translocation are associated with immune activation and systemic inflammation in virally suppressed PWH [12].

The role of fungal translocation on metabolic alteration and inflammation in HIV is unknown. Our objectives for this study were (1) to assess changes in BDG after initiation of ART with tenofovir disoproxil fumarate/emtricitabine (TDF/FTC)

with either atazanavir/ritonavir (ATV/r), darunavir/ritonavir (DRV/r), or raltegravir (RAL) in treatment-naïve participants and determine whether there are differences among the 3 regimens; (2) to assess whether baseline or changes in BDG are predictive of increases in visceral and overall adiposity and insulin resistance in PWH initiating ART; and (3) to assess whether BDG pre- and post-ART is associated with markers of monocyte and T-cell activation.

METHODS

A5260s was a cardiometabolic substudy of AIDS Clinical Trials Group (ACTG) A5257 in which HIV-infected ART-naïve participants ≥18 years of age with HIV-1 RNA ≥1000 copies/mL were randomized in an open-label fashion to receive standard doses of TDF/FTC with either ATV/r, DRV/r, or RAL. A5257 participants without known CVD or diabetes mellitus, uncontrolled thyroid disease, or use of lipid-lowering medications were eligible to enroll in A5260s. Further details have been previously published [13–16]. The analysis population in A5260s was restricted to a subset of participants who remained on their randomized treatment, achieved virologic suppression (HIV-1 RNA <50 copies/mL) by week 24, remained suppressed through week 96, and did not have treatment interruption of more than 7 days. Both A5257 and A5260s (ClinicalTrials.gov NCT00811954 and NCT00851799) were approved by the institutional review boards at participating institutions, and participants provided written informed consent.

Study Evaluations

Blood samples (fasting for ≥8 hours) were collected at baseline and weeks 4, 24, and 96. They were stored at –70ºC degrees and not previously thawed until analyzed in batches. Levels of intestinal fatty acid binding protein (I-FABP; R&D Systems, Minneapolis, MN, USA), ileal bile acid protein (I-BABP; Cloud Clone, Katy, TX, USA), and zonulin (ALPCO, Salem, NH, USA) were measured by enzyme-linked immunosorbent assay (ELISA) in the Dahms Clinical Research Unit at University Hospitals Cleveland Medical Center (Dr. McComsey, lab Director), whereas BDG (Mybiosource Inc., San Diego, CA, USA) was measured by ELISA in Dr. Funderburg's laboratory at Ohio State University, Columbus, Ohio.

Plasma biomarkers of inflammation were measured at the University of Vermont Laboratory for Clinical Biochemistry Research Lab (Burlington, VT, USA) and included high-sensitivity C-reactive protein (hsCRP) by nephelometry, D-Dimer by immunoturbidometric methods, and sCD14, sCD163, and IL-6 by enzyme-linked immunosorbent assay.

Peripheral blood mononuclear cells were collected and cryopreserved. T-cell activation was identified as the percentage of CD4+ or CD8+ cells expressing both human leukocyte antigen-D related (HLA-DR) and CD38.

Body composition measures occurred at baseline and week 96. Fat distribution was measured using whole-body dual-energy absorptiometry (DXA) and used to quantify total and trunk fat. Single-slice computed tomography (CT) scan was used to quantify VAT, subcutaneous abdominal tissue, and total adipose tissue. Scans were read at a central location, as previously described [6]. The homeostasis model assessment-insulin resistance (HOMA-IR) index was used to estimate insulin resistance [17].

Statistical Analysis

Non-normal biomarker data were transformed to the log10 scale for analysis; undetectable BDG results, reported as 0 pg/mL, were imputed as 1 pg/mL before transformation. Changes from baseline in BDG levels were summarized at weeks 4, 24, and 96 for each treatment arm using mean fold-changes and 95% confidence intervals; the analysis was done on the log10 scale and back-transformed for presentation. Wilcoxon rank-sum tests were used to contrast changes in biomarkers between treatment arms in a pairwise manner using a 2-sided 2.5% type I error rate; all other statistical tests used 2-sided 5% type I error rates. Associations between BDG levels and changes in adiposity and insulin outcomes were evaluated with linear regression models. Relationships between BDG and other biomarkers were assessed with Spearman's correlations. All analyses were conducted using SAS 9.4.

RESULTS

Baseline Characteristics and Disposition

Baseline demographic characteristics of the 328 participants from A5260s have been previously described [18]. A total of 231 (70%) participants included in the virologically suppressed population were included in this analysis; 67 participants in the ATV/r arm, 82 in the DRV/r arm, and 82 in the RAL arm. The baseline characteristics have been previously described [19]. Overall, 90% were male, the median age was 36 years, 55% were current smokers, the median body mass index was 25 kg/m^2, the median baseline CD4 cell count was 338 cells/μL, and the median HIV RNA was 4.56 log$_{10}$ copies/mL. There were no participants on antifungal medications or with active fungal infection. Baseline levels of BDG and body composition did not differ between treatment arms.

Changes in BDG After ART

As seen in Figure 1, BDG levels were slightly lower at week 4 compared with baseline in all treatment arms, with a mean fold-change of 0.57 (95% confidence interval [CI], 0.47 to 0.69). In a sensitivity analysis, which excluded the 20 participants who had BDG values of 0 pg/mL at week 4 (all of whom had detectable levels at baseline), the observed decrease remained, although slightly attenuated; the mean fold-change estimate was 0.83 (95% CI, 0.74 to 0.94).
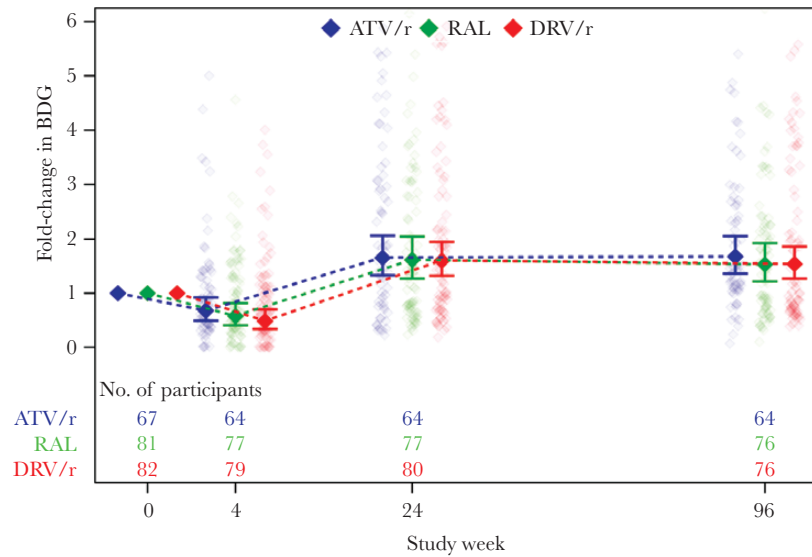
Figure 1. Mean fold-change and 95% confidence intervals are shown by treatment arm at each time point. Abbreviations: ATV/r, atazanavir-boosted ritonavir; BDG, β-d-glucan; DRV/r, darunavir-boosted ritonavir; RAL, raltegravir.

This decrease in BDG levels at week 4 was not sustained at later time points. Contrary to our hypothesis, increases were observed across all arms at weeks 24 and remained at week 96. Changes from baseline were similar in magnitude at weeks 24 and 96, suggesting that little additional change occurred between weeks 24 and 96. The mean fold-change was 1.62 (95% CI, 1.43 to 1.83) at week 24 and 1.57 (95% CI, 1.39 to 1.77) at week 96. Mean fold-changes in BDG were slightly higher among the ATV/r arm compared with the RAL and DRV/r arms across all study weeks, particularly at week 4. Differences were not statistically significant, however, at any time point ($P \geq .24$) (Table 1).

Baseline BDG did not correlate with CD4 cell count or viral load at baseline (($|r| < .1$; $P > .93$), and BDG levels did not correlate with CD4 at any time points on ART ($|r| < .1$; $P > .42$) Similarly, changes in BDG levels by week 4 did not correlate with changes in HIV-1 RNA by week 4 ($|r| = .01$; $P = .93$).

Associations Between BDG With Measure of Body Composition and Insulin Resistance

Linear regression models examined the relationships between BDG levels (baseline, week 4, week 24, and week 96) and percent change in adiposity outcomes at week 96 (Table 2). Associations between week 96 levels of BDG and changes in trunk fat and total fat at week 96 were observed. A 2-fold higher BDG level at week 96 was associated with an 8% increase in trunk fat and a 7% increase in total fat at week 96. These associations remained after adjusting individually for potential confounders including age, physical activity, smoking, alcohol and drug history, race, and sex.

In Spearman correlation analyses, there was no correlation between BDG level and HOMA-IR at weeks 0, 4, and 96 ($P \geq .36$). In addition, there was no correlation between change in BDG level and change in HOMA-IR at weeks 4 and 96 ($P \geq .73$).

Table 1. Relative Fold-Change in BDG From Baseline Between Treatment Arms

Treatment Comparison		Change From Baseline at		
		Week 4	Week 24	Week 96
ATV/r vs DRV/r	Mean (97.5% CI)	1.39 (0.79 to 2.42)	1.03 (0.74 to 1.44)	1.09 (0.79 to 1.50)
	P	.38	.71	.27
ATV/r vs RAL	Mean (97.5% CI)	1.17 (0.68 to 1.99)	1.03 (0.71 to 1.49)	1.09 (0.77 to 1.56)
	P	.65	.50	.24
RAL vs DRV/r	Mean (97.5% CI)	0.84 (0.48 to 1.48)	0.99 (0.70 to 1.40)	1.01 (0.72 to 1.41)
	P	.68	.69	.84

Pairwise treatment group comparisons of relative fold-change in BDG from baseline and respective study visit.

Abbreviations: ATV/r, atazanavir-ritonavir; BDG, β-D-glucan; CI, confidence interval; DRV/r, darunavir-ritonavir; RAL, raltegravir.

Table 2. Regression Estimates for Week 96 Percent Change in Adiposity Measures

Percent Change at Week 96		BDG Level at			
		Week 0	Week 4	Week 24	Week 96
Total fat	Estimate (95% CI)	1.54 (−2.24 to 5.32)	−1.80 (−3.86 to 0.27)	7.11 (−0.43 to 14.65)	6.89 (0.49 to 13.29)
	P	.42	.088	.065	**.035**
Trunk fat	Estimate (95% CI)	1.76 (−2.61 to 6.13)	−2.03 (−4.43 to 0.36)	9.27 (0.57 to 17.98)	8.05 (0.67 to 15.44)
	P	.43	.096	**.037**	**.033**
VAT	Estimate (95% CI)	2.88 (−4.22 to 9.98)	−0.17 (−4.13 to 3.78)	−3.63 (−17.67 to 10.41)	3.30 (−8.84 to 15.43)
	P	.43	.93	.61	.59
SAT	Estimate (95% CI)	1.28 (−4.14 to 6.70)	−0.36 (−3.32 to 2.59)	8.82 (−1.48 to 19.13)	4.65 (−4.58 to 13.87)
	P	.64	.81	.093	.32
TAT	Estimate (95% CI)	2.12 (−3.05 to 7.29)	−0.26 (−3.13 to 2.62)	6.49 (−3.77 to 16.76)	4.28 (−4.55 to 13.11)
	P	.42	.86	.22	.34
BMI	Estimate (95% CI)	0.46 (−0.62 to 1.53)	0.03 (−0.57 to 0.63)	1.27 (−0.88 to 3.42)	0.69 (−1.15 to 2.53)
	P	.40	.92	25	.47

Regression estimates are for unadjusted models only. Estimates are presented as per 0.3-log10 units increase, which is equivalent to a 2-fold difference.

Abbreviations: BDG, β-D-glucan; BMI, body mass index; CI, confidence interval; SAT, subcutaneous abdominal tissue; TAT, total adipose tissue; VAT, visceral adipose tissue.

Association Between BDG and Inflammatory and Gut Integrity Markers

At week 4, BDG was weakly associated with I-FABP ($r = .19$; $P < .01$) and zonulin ($r = .20$; $P < .01$). At week 96, there was a weak correlation seen between BDG and sCD14 ($r = .14$; $P = .04$) and D-dimer ($r = .18$; $P < .01$). In examining the changes from baseline, only a single marker, I-BABP, at week 24 showed a weak association with change in BDG level ($r = .14$; $P = .04$). No correlations were seen between BDG and any of the T-cell activation markers.

DISCUSSION

This is the first study to evaluate blood levels of BDG in HIV-infected participants before and after initiating a randomized ART regimen and its association with body composition, insulin resistance, and markers of inflammation and immune activation. We found that initially BDG significantly decreased after 4 weeks of TDF/FTC plus either ATV/r, DRV/r, or RAL. However, the acute decrease was reversed, with increases observed at week 24 and maintained through week 96. Modest associations were observed between BDG and I-FABP, zonulin, and sCD14, supporting the potential role of BDG as a biomarker of intestinal integrity and fungal translocation.

Fungal Translocation in HIV

Fungi are an integral part of the human microbiota that are dominated by yeast species [20, 21]. Data from the Human Microbiome Project investigated what constitutes a normal gut mycobiome and indicated that although fungal diversity is lower than bacterial diversity, the healthy gut contains *Saccharomyces*, *Malassezia*, and *Candida* [20], all of which produce the fungal polysaccharide BDG. In the general population, altered fungal microbiome (or mycobiome) has been shown to affect human health. For example, in inflammatory bowel disease, altered mycobiome is closely associated with disease activity [22, 23]. In the absence of fungal infections, blood levels of the fungal polysaccharide BDG are elevated after laparoscopic intestinal surgery and during hemodialysis, likely as a result of transient barrier damage in the gut after reduced blood flow [24]. It is clear that despite achieving virologic suppression, there is increased prevalence (~50%) of oropharyngeal colonization with fungal pathogens, most notably *Candida*, in PWH on ART [25]. In addition, the oral mycobiome in HIV has been defined; however, there are no data pre- and post-ART [26]. Thus far, studies in HIV have focused on bacterial translocation [27, 28] and few studies have investigated fungal translocation in HIV [29–31]. One smaller study investigated BDG pre- and post-ART [32] and showed that BDG remained stable 24 months after ART initiation in 21 participants with early HIV but increased in 14 participants in the absence of ART. Another larger case–control study of predictors of non-AIDS morbidity during ART-mediated viral suppression (n = 141 cases and n = 310 controls) also assessed BDG levels before and after ART (and before the non-AIDS morbidity event) [33]. Although that study did not report on time points during early ART, it did report significant declines in BDG levels from pre-ART to 1 year of ART-mediated viral suppression for both cases and controls, and higher BDG levels at year 1 predicted an increased risk of subsequent non-AIDS morbidity. Our study differs from this previous report in that BDG decreased in the first month of ART, followed by increases above baseline at week 24, elevations that persisted through week 96. It is unclear why these studies reached different conclusions, but we should note that we used a different ELISA kit to measure BDG in our study (Mybiosource) than was used in the earlier report (Fungitell, Cape Cod Associates), and, importantly, baseline CD4 counts were higher in our cohort.

For the first time, we compared the effect of protease inhibitors (PIs) and integrase inhibitors (INSTIs) on fungal translocation. We found no differences in plasma BDG levels between the 2 investigated PI-containing regimens, nor between the INSTI-containing regimen and the 2 PIs-containing regimens. In addition, in this analysis, we included only participants who were virologically suppressed by week 24 and remained as such throughout the 96 weeks of the study [34]. Inflammatory states such as HIV are known to induce disruption in intestinal integrity that persists despite ART [35]. We have previously shown that in A5260s, gut integrity, as measured by I-FABP, increased 1 month after ART initiation and then plateaued throughout the study period, with no difference between RAL and ritonavir-boosted PIs [35]. We showed that BDG is associated with gut biomarkers, specifically sCD14, a marker of bacterial translocation, I-FABP, a marker of enterocyte damage, and zonulin, a mediator of intestinal permeability. Based on our findings, we hypothesize that (1) the initial decrease in BDG may have been driven by the initial viral load suppression and increase in CD4 count that occurs with ART initiation and (2) the subsequent increase in fungal translocation may be a result of the ongoing gut barrier dysfunction that occurs despite ART. These findings may further support that early initiation of ART, but not duration of ART, may be more important in restoration of gut integrity and fungal translocation [32].

Contrary to our findings with BDG, we did not find evidence of bacterial translocation as measured by lipopolysaccharide binding protein (LBP) in this study [19]. This further supports our prior conclusion that unchanged LBP levels could be due to assay variability or lack of sensitivity of this marker compared with directly measuring the microbiome.

Fungal Translocation and Fat Accumulation and Insulin Resistance
Dysbiosis of the gut microbiota can cause changes in the host metabolism, energy storage, and modulation of gut hormones, which are associated with obesity, diabetes, and low-grade inflammation [36, 37]. Altered mycobiome has also been reported in obese individuals and found to be associated with higher amounts of body fat and insulin resistance, high blood pressure, and inflammation, as measured by CRP [38]. Although we failed to identify any associations between BDG and adiposity measures or HOMA-IR, we observed a correlation between postbaseline BDG levels and percent change in total fat and trunk fat, but not with changes in insulin resistance. These associations remained after adjusting for potential confounders such as demographics, substance use, and HIV-related factors. Our findings highlight the association between fungal translocation across the gut mucosa of PWH and excess fat accumulation.

Fungal Translocation and Inflammation
Similarly to LPS, BDG has been associated with inflammation and immune activation in PWH [12, 29, 32, 39, 40]. Contrary to

previous reports, we failed to observe any association between BDG and markers of monocyte, T-cell activation, or systemic inflammation at any time point. The differences in our results could be secondary to (1) gut marker assay variability, (2) the differences in study design and cross-sectional nature of these reported findings, (3) the small sample size in past studies and heterogeneity of the participants, specifically related to ongoing viremia and immune function, specifically baseline CD4 cell count.

Our study has several limitations. We assessed BDG, which is a surrogate marker of fungal translocation, and did not investigate the mycobiome of the participants. Additionally, BDG could have originated from other sites beyond the GI tract; however, without invasive fungal infections and high levels of epithelial gut damage, we believe that plasma levels of BDG likely reflect translocation from the gut. Our cohort is also relatively young and predominantly male; therefore, these results may not be generalizable to other PWH.

The strength of our analysis includes the longitudinal randomized nature of the study and the comprehensive evaluation of gut inflammatory and immune activation biomarkers.

CONCLUSIONS

Persistence of immune activation and metabolic complications despite long-term ART remains one of the biggest challenges in caring for PWH. We found that, despite viral suppression, fungal translocation decreased early, then subsequently increased similarly in regimens containing raltegravir or ritonavir-boosted PI, which may be the result of ongoing gut dysfunction. Increases in BDG were independently associated with changes in fat over 96 weeks, further supporting the complimentary role of fungal translocation and metabolic endotoxemia in HIV and related metabolic complications. Further longitudinal studies are warranted to understand the role of the mycobiome as a potential novel target for preventing metabolic complications in HIV.

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