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Abstract

Objective: Past correlational research has shown that minority stress has direct and indirect effects on the biology of sexual minority people. This pilot randomized control trial examined the potential of AWARENESS, a 9-session cognitive behavioral intervention to reduce intersectional minority stress, to alter gene expression related to immune function, inflammation, and HIV disease progression. **Methods:** Between 2016-2019, 25 sexual minority men living with HIV with recent substance use (n= 12 in AWARENESS and n = 13 in control) were enrolled, a subset with complete gene expression data among the 41 individuals within the parent randomized controlled trial. Blood samples were taken prior to the intervention, at the 9-week conclusion of the intervention, and at four months post-randomization, and leukocyte RNA was sequenced for all samples. The authors examined differential expression analyses of single genes and overrepresentation analysis of gene sets. **Results:** Neither AWARENESS nor the control condition were related to differential expression of single genes. Overrepresentation analysis suggested that AWARENESS was related to changes over time in gene expression in leukocyte RNA in 52 gene sets (q value < .05), many of which are related to immune function, while the active control condition was related to changes in gene expression among genes in only one gene set. When AWARENESS was compared to the control condition, four gene sets evidenced overrepresentation of genes reflecting change over time. **Conclusions:** This randomized controlled trial suggests that AWARENESS is associated with changes in gene expression, primarily focused on changes in genes associated with immune processes.

Keywords: Sexual and Gender Minorities, Gene Expression, Cognitive Behavioral Therapy

Public Significance Statement: This article describes change in gene expression pathways related to the immune system after a cognitive behavioral intervention for sexual minority men designed to reduce stress from stigma and discrimination. These results are important for public health because they shed light on how stigma and discrimination may impact health and how we may prevent related health disparities.

Introduction

Sexual minority (SM) men experience disparities in mental and physical health attributed to minority stress, including experiences of prejudice events, anticipation of prejudice events, concealment of identity status, and internalized stigma (Meyer, 2003), with additional HIV-status-related minority stress burden among sexual minority men living with HIV (Rendina et al., 2017). Systematic review shows that there are relationships between the different components of minority stress and multiple biological outcomes including sleep, respiratory health, cancer incidence, blood cell count, gene expression related to immune function, and cardiovascular outcomes (Flentje et al., 2019), that may be direct and/or indirect (*e.g., via* substance use, depression). Minority stress may impact immune function, inflammation, cardiovascular function, endocrine or hormonal function, and metabolic function (Flentje et al., 2019).

Immune function has specific clinical relevance to SM men living with HIV. Discrimination, concealment, internalized stigma, and discomfort in situations involving one's sexual orientation are related to lower CD4+ cell counts, faster progression to AIDS, and reduced life expectancy among SM men living with HIV (Bogart et al., 2013; Cole et al., 1997; Norcini Pala et al., 2015; Ullrich et al., 2003), suggesting a direct immune impact among SM men living with HIV.

Molecular mechanisms (*e.g.,* gene expression) may help us understand the biological impacts of minority stress *and* identify interventions that may reverse the impacts of minority stress. Gene expression has the potential to track response to behavioral interventions by elucidating the gene pathways and downstream processes, and potentially reversing inflammatory mechanisms of stress (*e.g.,* Buric et al., 2017 for a systematic review of mind-body interventions). Minority stress among SM living with HIV is related to molecular signatures in gene expression (Flentje et al., 2018), accelerated epigenetic aging (Ghanooni et al., 2022), and trajectories of immune-relevant longitudinal biomarkers in plasma such as depletion of tryptophan (Vincent et al., 2021), alteration of these signatures

through intervention would enable experimental testing of the underlying biology of minority stress to help us better understand the molecular impacts of minority stress through their reversal.

This pilot study examined changes in gene expression in response to AWARENESS: a 9-session cognitive behavioral intervention designed to reduce minority stress related to intersecting identities among sexual minority men living with HIV (described in detail in Flentje, 2020). We expected to find differential expression of single genes and overrepresentation of these genes in sets of genes known to work together (*i.e.,* gene sets) in response to the intervention, and relatively few changes in an active control intervention. Given prior work (Flentje et al., 2019), we anticipated that genes and gene sets evidencing change in response to AWARENESS would be related to immune function, inflammation, and HIV disease progression.

Methods

We followed CONSORT guidelines (Supplemental Material 1) for this pre-registered trial (clinicaltrials.gov, NCT03143205). Datasets generated during the current study are sensitive but are available from the corresponding author on reasonable request. Trial procedures are described in Flentje et al. (2022). Participants were recruited from the San Francisco Bay Area through flyers at health clinics, online (*e.g*., Craigslist), and through snowball sampling from 2016-2018. Inclusion criteria were: 18 years or older, fluent in English, able to consent, a cisgender sexual minority man (*i.e.,* male sex assigned birth, identifies as male, and sexual activity with a man in the three years prior or identification as gay or bisexual), documentation of HIV-positive status, and at least one occasion of drinking five or more drinks in a single setting or of using an illicit substance in the previous three months. Exclusion criteria included current: participation in substance use treatment, meeting criteria for a severe substance use disorder, or symptoms of schizophrenia or bipolar disorder while not using substances. This study was approved by the Institutional Review Board of the University of California,

San Francisco.

Participants were randomized to nine sessions of AWARENESS or an attention-matched control condition consisting of neutral writing exercises and questionnaires with a study interventionist. Intervention sessions were administered by a trained interventionist at a community clinic. Assessment and blood collection took place just prior to the first session, after the ninth/final session, and four months post-randomization by a blinded assessor. Participants were compensated $50 for assessment visits and $20 for intervention sessions. The parent randomized controlled trial had a final sample size of $N = 41$ ($n = 21$ in AWARENESS and $n = 20$ in the control condition; Flentje et al., 2022). For this analysis, participants were included if they had three available time points of RNA sequencing data.

Measures

Study measures (Supplemental Material 2) are reported in Flentje et al., 2022. HIV viral load was measured using Abbot Realtime HIV Viral Load and Roche assays (lower limit 40 and 20 copies per mL, respectively) and is reported undetectable at <50 copies per ml. A visual analog scale (ranging from 0-100) measured antiretroviral medication adherence (Giordano et al., 2004).

RNA sample preparation, sequencing, and analysis

Total RNA was isolated from samples collected into PAXgene tubes using a PAXgene blood miRNA kit (Qiagen). An RNA clean-up kit (Zymo Clean and Concentrator-5) optimized RNA for sequencing. Library preparation used Poly-A enrichment. RNA was sequenced by the University of California, Davis Genomics Core on a NovaSeq system at a depth of 35 million reads, 150 base pair paired-end reads, in a single lane. Expanded details on quality control procedures can be found in Supplement 3. Differential expression analyses of single genes were conducted via paired analysis using the DESeq2 package v1.32.0 (Love et al., 2014) using a Benjamini-Hochberg false discovery rate (FDR) q value (q) of .05. Outliers were examined using gene-count plots. Differential expression analyses were run three ways: 1) comparing change over time within the AWARENESS condition, 2)

comparing change over time within the control condition, and 3) comparing change over time between the AWARENESS and control conditions. Overrepresentation analyses using Clusterprofiler v 4.0.5 (Wu et al., 2021; Yu et al., 2012) examined gene sets that evidenced enrichment by entering the subset of genes from the differential expression analysis that had an unadjusted p <.05; results were interpreted at an FDR q value <.05. Gene sets were defined by the Gene Ontology (GO) database (Gene Ontology Consortium, 2021); enriched gene sets were queried through tools available related to their databases (Binns et al., 2009) and by looking in the literature to identify if these gene sets had emerged in studies of immune function, inflammation, or HIV disease progression (as identified in Flentje et al., 2019), focusing on the top 10 statistically significant results (*i.e.,* smallest q values). Visualizations are described and provided in Supplement 3.

Results

Participants

Among 41 participants included in the parent pilot randomized controlled trial, 26 had RNA sequencing data at all three time points; one participant was removed during outlier checks (see Supplement 3). Twenty-five participants were included for analysis (75 total samples across the time points). Table 1 contains demographic and clinical information.

Differential Gene Expression

No single genes evidenced differential gene expression within AWARENESS over time, control over time, or between AWARENESS and control over time (q>.05 for all). For overrepresentation analysis: 861 genes in AWARENESS, 278 genes in control, and 499 genes in AWARENESS versus control were retained due to unadjusted p<.05. In gene set analyses, AWARENESS had overrepresentation of genes evidencing change over time within 43 gene sets in the GO Biological Processes database, 7 gene sets in the GO Cellular Component database, and 2 gene sets in the GO Molecular Function database (52 total gene sets, see Supplement 4 for complete list). Within the biological processes database, most of

the gene sets identified were related to immune function: 21 gene sets were connected to humoral immune response mediated by circulating immunoglobulin and 10 gene sets were connected to cell regulation (detail in Supplement 3). In the top 10 gene sets in the Biological Processes database, 8 were nested within processes related to immune response. The top 3 were the humoral immune response mediated by circulating immunoglobin pathway (GO: 0002455), complement activation, classical pathway (GO: 0006958), and immunoglobulin mediated immune response (GO: 0016064), which have been associated with HIV disease progression (Hossain et al., 2021). We examined the 12 genes in these pathways with the smallest *p* values in differential expression analyses (all $p < .01$) and generated gene count boxplots (Supplement 3, Figure 4) to further examine when change was occurring within the single genes in these gene sets. Visual examination of these boxplots suggests that the largest changes are observed from baseline to 9 week follow-up, and that changes typically remain at 4 month follow-up or regress slightly towards baseline values, but remain different from baseline. B cell mediated immunity (GO: 0019724) and complement activation, classical pathway (GO: 0006958) have been implicated in aging (de Magalhães et al., 2009; Li et al., 2018). The top 5 gene sets in the biological process database tended to be downregulated over time (plot and results in Cellular Components and Molecular Function in Supplement 3). The control condition had one gene set with overrepresentation of genes that evidenced changes over time (GO: 0019814, immunoglobulin complex in the GO Cellular Component database, $q=0.030$), also overrepresented in AWARENESS. When comparing AWARENESS to the control condition, there were four gene sets that evidenced differences (Cellular Component database, detail in Supplement 3).

Discussion

This study reflects an experimental testing of the underlying biology of minority stress. Our results suggest that there is a biological signal associated with AWARENESS, an intervention aimed at undoing the impacts of minority stress by

promoting new ways of recognizing and coping with minority stress. Specifically, we saw an overrepresentation of genes in 52 gene sets that evidenced change over time in the AWARENESS condition and four gene sets that contained overrepresented genes in AWARENESS versus control over time, with only one gene set evidencing change over time in the control condition. Within this study, we did not observe differential expression of single genes, perhaps due to the sample size. However, our observation of the overrepresentation of sets of genes suggests changes in the coordinated expression of genes, expected to be more instrumental in relation to changes in overall biological processes.

In a prior study, we reported on the self-report outcomes within this trial (Flentje et al., 2022). Within that pilot study, the self-reported outcomes (*i.e.,* minority stress, depression, anxiety, substance use, alcohol use, posttraumatic stress disorder symptoms, and antiretroviral therapy adherence) did not differ between the AWARENESS and control conditions over time. This study suggests that it is possible that molecular outcomes like gene expression may detect changes that are not observable through self-report measures. The importance of these changes observed in gene expression remains to be seen, though the high frequency of change we observed in gene sets implicated in immune processes suggests that these changes may reflect meaningful changes in immune function. A prior study of a mindfulness intervention versus a control condition found that both interventions were perceived by participants to have lowered stress, but only the active mindfulness intervention resulted in a significant reduction in pro-inflammatory cell gene expression (Dutcher et al., 2022). Taken together, these findings suggest that biological markers may have more precision in capturing change than self-report, which may be prone to reporting biases and placebo effects.

Immune processes emerged as prominent in the gene sets that evidenced change in the AWARENESS intervention, an important consideration for all people, especially people living with HIV. Minority stress is hypothesized to directly impact

immune function (Christian et al., 2021; Flentje et al., 2019), which appears consistent with our results here. The observed changes over time in gene sets related to immune processes suggest that AWARENESS may be altering pathways impacted by minority stress. Additional investigation of AWARENESS is warranted in a larger sample to identify the replicability of results and strength of effects.

There are several limitations, including that we could only analyze data from participants with usable RNA at all three time points which resulted in a small sample size that may produce some unstable or unreplicable findings. Preexisting differences between groups may impact results, though it is unlikely that all of these preexisting differences are on the causal pathways for the gene set differences observed here. This study only included sexual minority men living with HIV, thus, results cannot be generalized to other LGBTQIA+ people. A larger trial will be needed to identify which, if any, results replicate and could be expanded to other sexual and gender minority groups. Further, minority stress theory needs careful integration into clinical interventions. Our results here suggest that the intervention *may* address minority stress constructs by inferring the effects on gene expression outcomes, though these changes could be due to other intervention effects.

It has been challenging to identify the specific biological signature of minority stress; results from other studies examining biological outcomes have often had inconsistent results (Flentje et al., 2019). A cognitive behavioral intervention specifically targeting minority stress has the potential to help us understand underlying biological mechanisms of minority stress through reversal of these mechanisms. Here, we see a strong signal with our AWARENESS intervention that is not observed in our control condition, suggesting that we may be on the path to better understand how minority stress may impact the biological functioning of an individual. To support the health of sexual *and* gender minority communities we must move beyond descriptive cross-sectional studies of minority stress and mental health conditions and begin to understand *how* minority stress may impact health and what we can do to undermine that impact.

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Table 1 Baseline demographic and clinical information of 25 cisgender sexual minority men living with HIV who participated in the AWARENESS pilot RCT and had RNA sequencing data

	AWARENESS condition *n* = 12	Attention matched control *n* = 13
Sexual orientation		
Gay	12 (100%)	13 (100%)
Age M (SD), Range	53.9 (7.7), 36.46-66.95	52.7 (13.1), 24.63-73.61
Race		
Asian	1 (8%)	0%
Black/African American	1 (8%)	3 (23%)
Filipino	0 (0%)	0 (0%)
Multiracial	1 (8%)	1 (8%)
Native American	1 (8%)	1 (8%)
Prefer not to answer	1 (8%)	0 (0%)
White	7 (58%)	8 (62%)
Ethnicity		
Hispanic/Latino	2 (17%)	2 (15%)
Income		
< $10,000	9 (75%)	5 (39%)
$10,000-$20,000	0 (0%)	3 (23%)
$20,000-$30,000	3 (25%)	0 (0%)
$30,000-$40,000	0 (0%)	2 (15%)
$40,000-$100,000	0 (0%)	1 (8%)
>$100,000	0 (0%)	2 (15%)
Education		
High school graduate	3 (25%)	1 (8%)
Trade/technical school	1 (8%)	0 (0%)
Some college	3 (25%)	1 (8%)
2 -year college degree	1 (25%)	3 (23%)
4-year college degree	3 (25%)	5 (39%)
Graduate or professional degree	1 (8%)	3 (23%)
Baseline ASSIST		
Low risk for alcohol or another drug	4 (33%)	3 (23%)
Moderate risk for alcohol or another drug	7 (58%)	9 (69%)
High risk for alcohol or another drug	1 (8%)	1 (8%)
Baseline AUDIT $\geq$ 8[a]	1 (8%)	3 (23%)
Baseline PHQ-9 $\geq$ 10[a]	2 (17%)	3 (23%)
Baseline GAD-7 $\geq$ 10[a]	2 (17%)	1 (8%)
Baseline Viral load undetectable (<50 copies/mL)[1]	10 (83%)	12 (92%)
ART medication adherence $\geq$ 95%	9 (75%)	8 (73%)
IHP-R M (SD), range	1.35 (0.59), 1-2.60	1.53 (0.80), 1-3.75

CARS M (SD), range	13.5 (4.33), 9-21	11.62 (5.30), 5-20

[1] Viral load data was missing for 1 participant at baseline.