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**Neurotoxicity with high dose disulfiram and vorinostat used for HIV latency reversal**

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**Running head:** Combination HIV latency reversal

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## Abstract

**Objective:** To examine whether administering both vorinostat and disulfiram to people with HIV (PWH) on antiretroviral therapy (ART) is safe and can enhance HIV latency reversal

**Design:** Vorinostat and disulfiram, can increase HIV transcription in people with HIV (PWH) on antiretroviral therapy (ART). Together these agents may lead to significant HIV latency reversal**.**

**Methods:** Virologically suppressed PWH on ART received disulfiram 2000mg daily for 28 days and vorinostat 400mg daily on days 8-10 and 22-24. The primary endpoint was plasma HIV RNA on day 11 relative to baseline using a single copy assay. Assessments included cell-associated (CA) unspliced (US) RNA as a marker of latency reversal, HIV DNA in CD4+ T-cells, plasma HIV RNA and plasma concentrations of ART, vorinostat and disulfiram.

**Results:** The first two participants (P1 and P2) experienced grade 3 neurotoxicity leading to trial suspension. After 24 days, P1 presented with confusion, lethargy, and ataxia having stopped disulfiram and ART. Symptoms resolved by day 29. After 11 days, P2 presented with paranoia, emotional lability, lethargy, ataxia and study drugs were ceased. Symptoms resolved by day 23. CA-US RNA increased by 1.4- and 1.3-fold for P1 and P2 respectively. Plasma HIV RNA was detectable from day 8-37 (peak 81 copies/mL) for P2 but was not increased in P1 Antiretroviral levels were therapeutic and neuronal injury markers were elevated in P1.

**Conclusions:** The combination of prolonged high dose disulfiram and vorinostat was not safe in PWH on ART and should not be pursued despite evidence of latency reversal.

**Key words:** Latency reversal, disulfiram, vorinostat, neurotoxicity, clinical trial

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## Introduction

A major barrier to a cure for HIV is the persistence of long lived and proliferating latently infected cells. [1] One strategy to reduce latently infected cells in people with HIV (PWH) on antiretroviral therapy (ART) is to use latency-reversing agents (LRAs) to induce virion production leading to virus-mediated cell lysis or immune-mediated killing.

Clinical trials of LRAs in PWH on ART, have evaluated histone deacetylase inhibitors (HDACi) vorinostat, [2, 3] romidepsin [4] and panobinstat [5] and the anti-alcoholism drug disulfiram [6, 7]. These increase cell-associated (CA) unspliced (US) HIV RNA and in some studies plasma HIV RNA, consistent with latency reversal but did not reduce the frequency of latently infected cells, potentially because of insufficient potency with single-agents as suggested by *in vitro* studies [8]. HDACi and disulfiram reverse HIV latency through different mechanisms [9] and in *ex vivo* studies both were shown to increase CA US HIV RNA and had an additive effect when combined together.

In PWH on ART using 500mg, 1g or 2g of disulfiram for 3 days, disulfiram was well tolerated and increased CA US HIV RNA at all doses and plasma HIV RNA at the 2g dose. Additionally, disulfiram has been safely used clinically at daily doses ranging from 500 mg-3g [10] and up to 6g [11] in individuals with alcohol dependence. We previously also showed that the HDACi vorinostat was tolerable at 500mg/day in PWH on ART, and increased CA US HIV RNA [2] with no adverse clinical or virological effects over two years [12]. As disulfiram and vorinostat target different pathways that maintain HIV latency, have been evaluated alone in PWH on ART, have additive effects on HIV transcription *in vitro* and *ex vivo* and have acceptable safety profiles, we hypothesised that combining these drugs for PWH on ART would be safe and provide significant latency reversal *in vivo*.

## Methods

We performed a clinical trial in PWH receiving ART with plasma HIV RNA <50 copies/mL for at least 3 years, a CD4 count >350 cells/µL and able to abstain from alcohol. The Alfred Human Research Ethics Committee approved the study (No. 106/17) which was registered on clincialtrials.gov (NCT03198559) and written informed consent was obtained. Participants received disulfiram 2000mg daily for 28 days, plus vorinostat 400mg, daily on days 8-10 and 22-24 (Figure 1A) and were monitored for adverse events [13].

## Virological analyses

CA US, multiply spliced (MS) HIV RNA and total HIV DNA were quantified in total CD4+ T-cells [2] and HIV RNA was quantified to a single copy/mL [14]. Expression of p24 protein was measured the single-molecule-array platform from frozen PBMC [15] and histone (H4) acetylation was assessed using ELISA. [16]

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Additional methods describing, cytokine and drug analysis, gene expression, plasma markers of central nervous system injury, molecular markers of transcriptional activity and statistical methods are available in supplementary material, http://links.lww.com/QAD/C329.

**Results**

Two individuals were enrolled and both experienced Grade 3 neurotoxicity leading to suspension of trial enrolment.

Participant 1 (P1) was a 67 year-old male receiving abacavir/lamivudine/dolutegravir. His CD4 count was 762 cells/μL with HIV RNA <20 copies/mL for 15 years. He had treated hypertension and hypercholesterolemia. On D10 he reported fatigue and anorexia and stopped both study drugs. He commenced disulfiram again on D11 and on D16 reported no adverse effects. On D24 he reported feeling cold, myalgias, lethargy, mild confusion and was ataxic. He had ceased all medication including study drugs and ART for possibly up to a week since D16. He was admitted to hospital and had mildly elevated liver enzymes and stable renal function. Cerebrospinal fluid revealed elevated protein 0.59 g/L and (normal 0.15-0.4 g/L). Neuroimaging, revealed a left sigmoid sinus thrombosis, chronic left vertebral artery occlusion and old right cerebellar infarct. Neurology review noted it was possible the sinus thrombosis could explain his symptoms, recommending 3 months anticoagulation then lifelong aspirin. His symptoms resolved and was discharged on D29. Due to the sinus thrombosis and symptom onset after commencing disulfiram the events were deemed possibly related to disulfiram, grade 3 severity and a serious adverse event (SAE).

Participant 2 (P2) was a 61 year-old male receiving tenofovir alafenamide/emtricitabine and raltegravir. His CD4 count was 1085 cells/μL and plasma HIV RNA <20 copies/mL for 7.8 years. His history included skin carcinomas, osteoarthritis, peripheral neuropathy and pulmonary embolus. He reported lethargy, dysgeusia and diarrhea from D2-8 and a close contact with gastroenteritis possibly causing his diarrhea but in retrospect was likely related to disulfiram. On D11 he was fatigued, emotionally labile, anxious, displaying pressured speech, paranoid ideation and ataxia. He was admitted to hospital with last study drug doses on D10. Investigations revealed normal CT brain and stable renal and hepatic function. Lumbar puncture was not performed. His paranoia resolved by D15, he was discharged on D23 and all symptoms resolved by D28. The events were reported as probably related to disulfiram and possibly related to vorinostat or an unexpected drug interaction between disulfiram and vorinostat. They were grade 3 in severity and an SAE leading to treatment discontinuation and enrolment ceased

**Virological effects**

The fold change increase in CA US HIV RNA from baseline to D8 was 1.4- and 1.3-fold in P1 and P2 respectively which persisted in P1 but declined in P2. Plasma HIV RNA was undetectable in P1 but rose from 2 to 81 copies/mL on D21 in P2. Therapeutic antiretroviral drug levels confirmed ART compliance with P2 also having observed therapy in hospital

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from day 11-23. Fold change in HIV DNA compared to baseline for P1 and P2 was 0.7- and 0.6-fold respectively and there were no consistent patterns of change in p24 expression (Figure 1B).

**Drug levels**

Plasma concentrations of antiretrovirals and vorinostat were therapeutic, except for antiretroviral levels in P1 on D21 when all medications had stopped (Table S1, http://links.lww.com/QAD/C329). In P2, disulfiram and carbamathione levels were detectable when disulfiram was taken but below the limit of quantification (Table S1, http://links.lww.com/QAD/C329). There was increased histone acetylation in P2 after 3 vorinostat doses, but no increase in P1 after two doses (Figure 1B).

**Significant changes in gene expression following vorinostat**

Principal component analysis (PCA) of gene expression revealed a similar directional shift between P1 and P2 in clustering at different time points (Figure 2a). At the "end of study", the transcriptomic profile was close to baseline for P2 but remained distinct from baseline for P1. Many differentially expressed genes (DEG) were identified at D11 after participants had received both drugs with 884 genes significantly upregulated and 170 genes significantly downregulated compared to baseline (Figure 2b). Comparing baseline to end of study there were no significant DEG. Reactome analysis detected changes in gene pathways between D8 and D11, which we conclude were due to vorinostat, including upregulation of chromatin organization, signal transduction, the immune system and initiation of gene transcription (Figure 2c). Gene pathways in both participants reverted towards baseline by the end of study but there were still significant changes present at this time point. CDK9 and NF-kB activation are factors associated with HIV transcription [17]. In P1, CDK9 phosphorylation increased at D11 with a later decline in NFkB, while P2 had less prominent changes (Figure S2, http://links.lww.com/QAD/C329)

**Changes in cytokines suggesting innate immune activation**

Upregulation of cytokine expression associated with innate immune activation including an increase in IL6, IL1, IL8 and IL13 was seen in P1. Less prominent changes were seen in P2 and the end of treatment sample clustered more closely with baseline as compared to P1 (Figure S1, http://links.lww.com/QAD/C329).

**Plasma neurofilament light chain**

Plasma neurofilament light chain protein (NfL) and glial fibrillary acidic protein (GFAp,) can be elevated in neurological disease including HIV [18, 19]. Baseline plasma NfL was similar to previously reports in PWH [18] and GFAp levels were normal [20]. NfL and GFAp levels rose above the 90$^{th}$ centile and returned to baseline in P1 who also had dural sinus thrombosis but did not change in P2 (Figure S2, http://links.lww.com/QAD/C329)

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## Discussion

We aimed to determine the effects of combining prolonged high dose disulfiram and vorinostat on reversing HIV latency but observed neurotoxicity in the first two participants that terminated the trial. The neurotoxicity was consistent with disulfiram toxicity, although we could not detect high plasma disulfiram levels. An alternative explanation is an unanticipated interaction between disulfiram and vorinostat. We also observed an increase in CA US HIV RNA in both participants and plasma HIV RNA in one participant which were modest, but consistent with latency reversal. Despite the potential for increased HIV transcription with this combination it should not be pursued due to the neurotoxicity.

The recommended maintenance dose of disulfiram is 125-500mg per day [21]. but is titratable against clinical response. Up to 25% of individuals receiving over 500mg/day can experience neurotoxicity [22]. The severity of this toxicity has been linked to duration of dosing and the maximum dose with increased likelihood of toxicity when dosing up to 3g daily for 2 weeks [23]. The presentation of delirium, paranoia, emotional lability and ataxia in both participants is clinically consistent with prior descriptions of disulfiram toxicity [21]. Notably, neurological symptoms started before vorinostat in both individuals and these neurotoxic effects have not been described with vorinostat [24]. In our previous trial, with dosing of disulfiram at 500mg, 1g or 2g for 3 days, there were 11 mild neurological adverse events in the 2g cohort that resolved [6] and disulfiram was easily detectable in plasma at this dose. Our ability to only detect low disulfiram levels in this study could be explained by the fact that we used a different method to quantify the drug, however, all positive controls clearly detected the drug. Alternatively, in this study compared to our previous study, we observed a longer time between sample collection and isolation of plasma, potentially leading to degradation of disulfiram in the samples [6].

We found that the transcriptomic profile of disulfiram plus vorinostat effected gene expression but there were limited changes after disulfiram monotherapy. Although the interpretation is limited with two participants, the increased gene expression and up-regulation of gene pathways involved in functions such as chromatin organization are consistent with the mechanism of action of vorinostat, an epigenetic modifying agent. It would be interesting to see whether exposure to disulfiram affects host response to vorinostat. Our previous trial of vorinostat monotherapy used a microarray sequencing method [2] which is different to this study utilising RNA-seq so comparison is not possible between the two data sets.

The more significant changes in cytokines and markers of neurological damage were observed in P1, but it is unclear what was driving the innate immune activation in this participant. One option is HIV itself, following HIV latency reversal. HIV RNA is a potent activator of innate immunity through binding to TLR7 and TLR8 [25] but we think this is unlikely given the modest latency reversal. Although vorinostat can activate NFkB, it is associated with reducing inflammation [26, 27] and attenuated IFN-γ-induced neurotoxicity

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in an animal model [28]. We could find no reports of disulfiram increasing immune activation.

In conclusion, the combination of continuous high dose disulfiram and vorinostat led to latency reversal but also significant neurotoxicity. The clinical presentations were consistent with disulfiram toxicity, although we cannot exclude an unexplained interaction between disulfiram and vorinostat. Due to the severity of these adverse events, this combination should not be pursued in clinical trials targeting persistent HIV.

**Contributors**

SRL, JM and TAR conceived of and designed the study and developed the study protocol. SRL, JM and JSYL provided clinical oversight of the study. VE, AS, AD and ST coordinated trial sample processing and laboratory procedures. JM and JSYL enrolled patients in the study and performed follow-up. VE, JMZ, AS, AD and ST performed virological and immunological analyses. SP and KF performed ultrasensitive measurement of plasma HIV RNA. HZ and KB performed measurements of plasma biomarkers for brain injury. DB performed antiretroviral drug levels. NNB and CJSH performed analysis of disulfiram and carbamathione plasma levels. GW, PZ, and BH performed p24 and histone acetylation assays. SRL, JM, TAR, and JS performed data interpretation. The manuscript was drafted by JM, and SRL. All authors reviewed and provided input to the manuscript and approved the final version.

**Disclosures**

HZ is a Wallenberg Scholar and served at scientific advisory boards for Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics and CogRx, has given lectures in symposia sponsored by Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program. KB has served as a consultant, at advisory boards, or at data monitoring committees for Abcam, Axon, Biogen, JOMDD/Shimadzu. Julius Clinical, Lilly, MagQu, Novartis, Roche Diagnostics, and Siemens Healthineers, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program.

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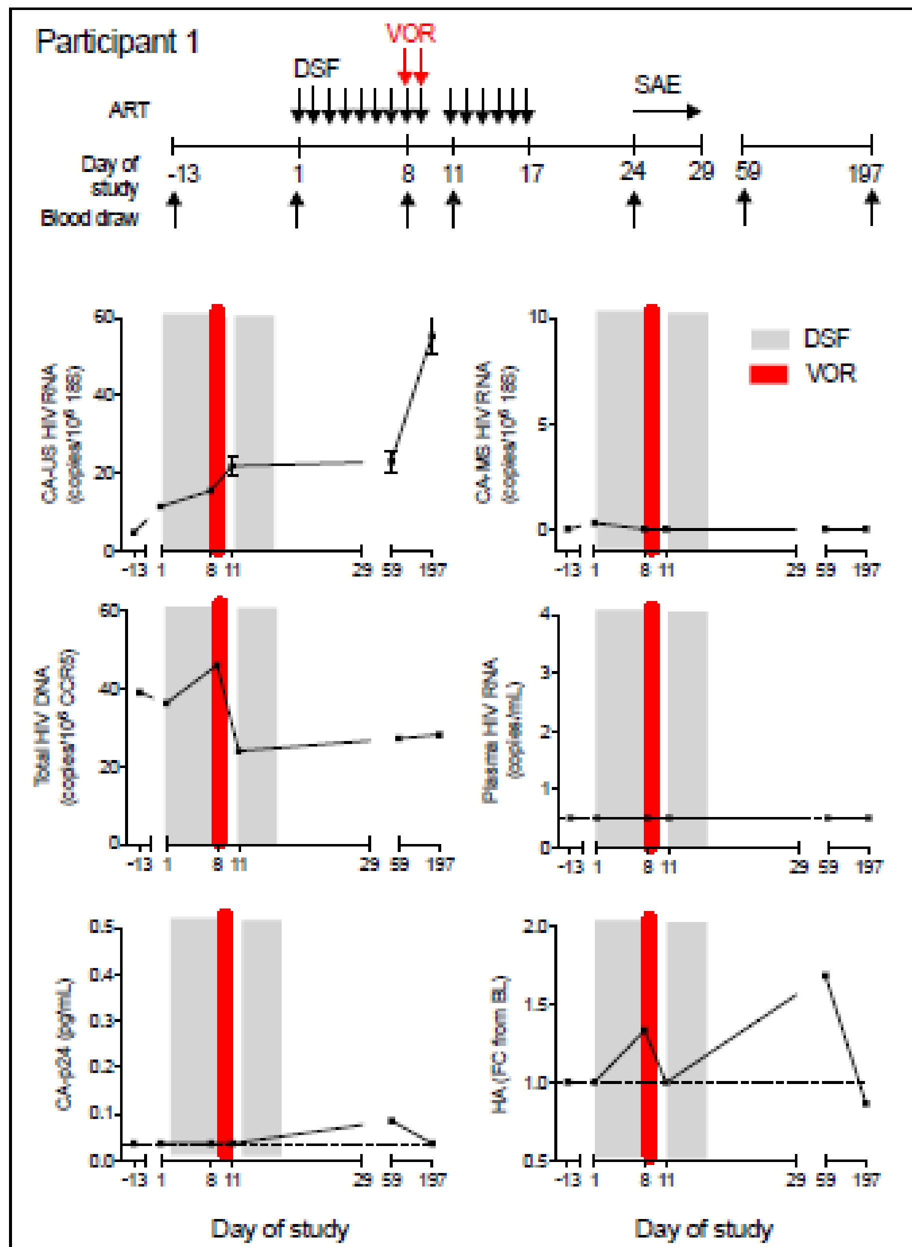
**Figure 1. Clinical and laboratory parameters following disulfiram and vorinostat. A.** Clinical trial design of arospective single arm study of 28 days of disulfiram 2 g/ day with intermittent vorinostat in PWH on suppressive ART. Time of blood collections is shown by green arrows. B. Participant details from P1 and P2 showing the timing of administration of study drugs including disulfiram (DSF) and vorinostat (VOR), onset of severe adverse effects (SAE) and collection of samples in both participants who were also on antiretroviral therapy (ART). Cell associated unspliced (CA-US) RNA, CA-multiply spliced (MS) RNA, total HIV DNA and plasma HIV RNA were quantified by PCR. p24 protein in blood PBMC lysate was quantified using the single molecular array (Simoa) platform. Histone acetylation (HA) was quantified by ELISA and is shown as fold change (FC) from baseline (BL). For all graphs, grey shading indicates time on DSF and red shading indicates time on DSF and VOR.

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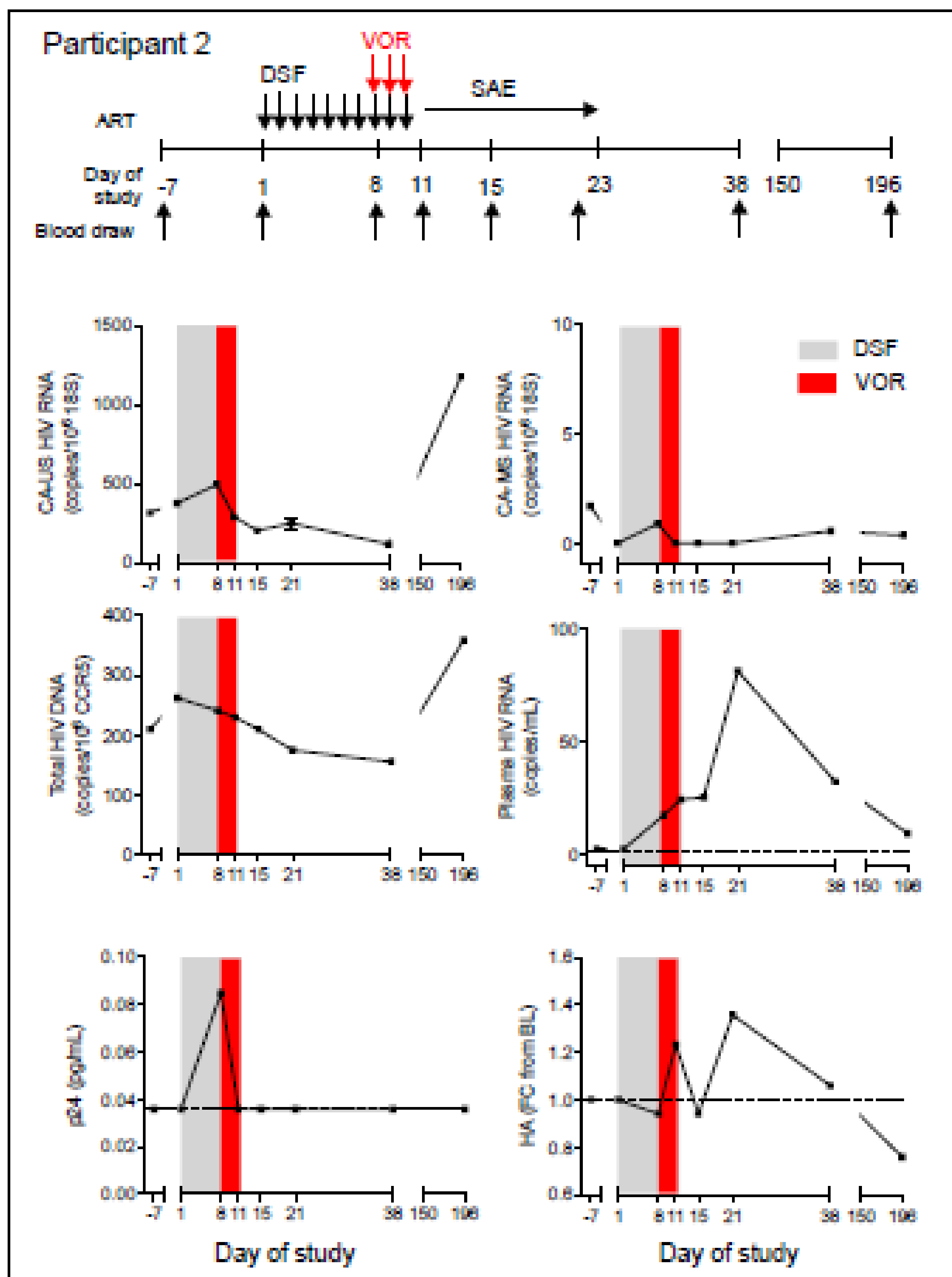


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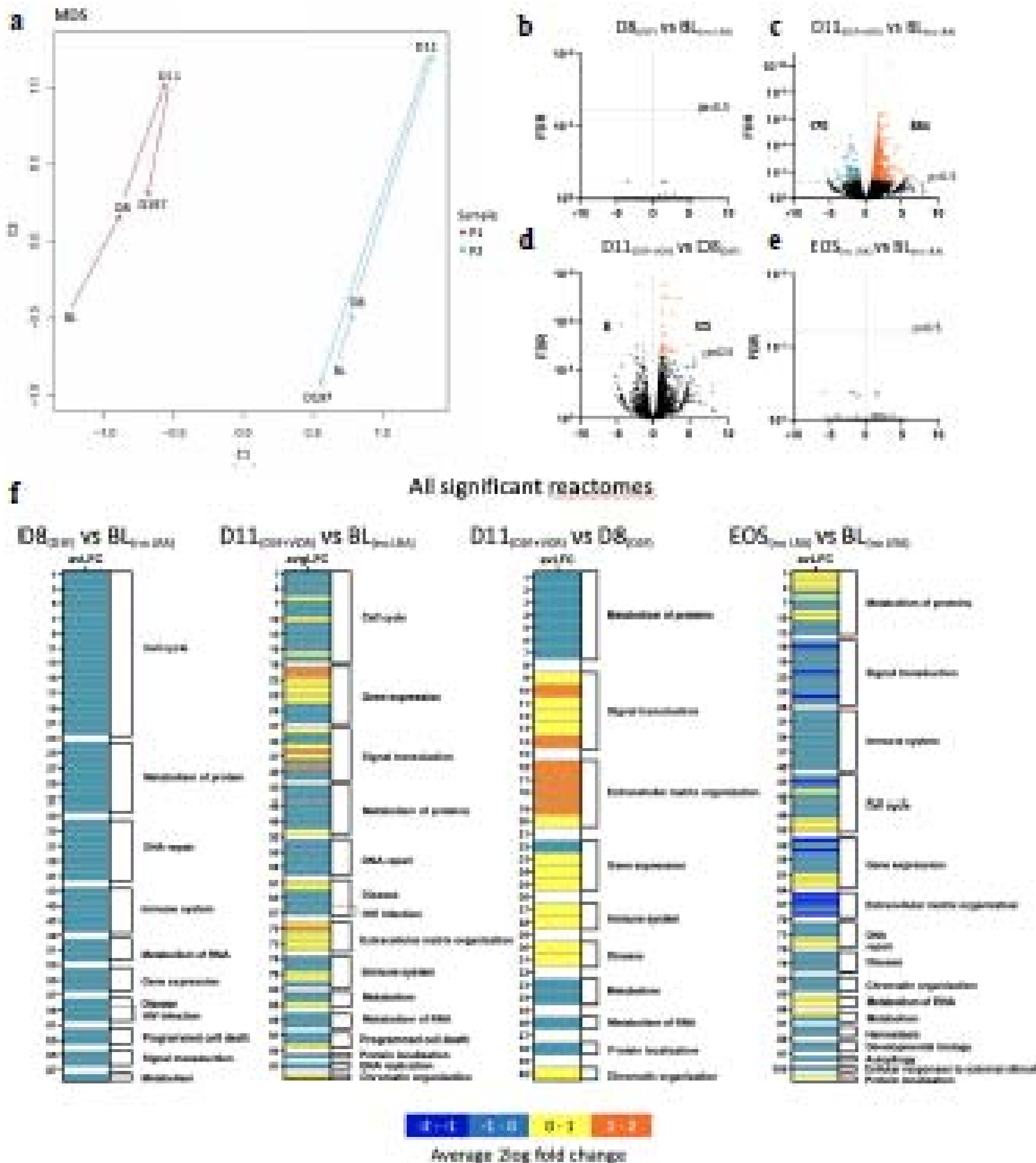
**Figure 2: Significant transcriptomic changes observed following disulfiram and vorinostat.** Bulk RNA sequencing was performed in PBMCs from both participants at baseline (BL), day (D) 8, D11 and at end of study (EOS, D197). A. Multidimensional scaling plot demonstrating differing transcriptomic profiles for P1 (red) and P2 (blue). B-E. Volcano plots showing gene expression when comparing two including baseline (BL), D8 (disulfiram only) D11 (disulfiram and vorinostat) and EOS. Orange dots show upregulated and blue dots downregulated genes. F. Reactome analysis is shown as gene families that are significantly up and down regulated when two time points are compared. The magnitude of the change is reflected by the color scale.

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