Immunoproteome of anti-TB drug associated DRESS in HIV TB co-infection

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Declaration

I, Chloe Buck, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, or being, or is to be submitted for another degree in this or any other university.

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— List of abbreviations

HIV	Human Immunodeficiency Virus
ADR	Adverse drug reaction
IM-ADR	Immune-mediated adverse drug reactions
CADR	Cutaneous adverse drug reactions
SCAR	Severe cutaneous adverse drug reactions
SJS/TEN	Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN)
DRESS	Drug reaction with eosinophilia and systemic symptoms
T_{REG}	Regulatory T cells
HLA	Human Leukocyte Complex
MHC	Major Histocompatibility Complex
TCR	T-cell receptor
CTL	Cytotoxic T-cell lymphocyte
IL-X	Interleukin-X
TNF-α	Tissue Necrosis Factor alpha
IFN-γ	Interferon-gamma
NK	Natural Killer
Th1	T helper type 1
Th2	T helper type 2
PPE	Papular pruritic eruption
ART	Antiretroviral therapy
TB	Tuberculosis
FLTB	First-line Tuberculosis drugs

PBMC	Peripheral Blood mononuclear cell
μm	Micromolar
mM	Millimolar
LC-MS/MS	Liquid-Chromatography Mass Spectrometry
BCA	Bicinchoninic Acid
MS	Mass Spectrometry
HCD	higher-energy collision dissociation
LFQ	Label-free quantitation
Q-TOF	Quadrupole Time of Flight
MAP	Multi analyte profiling
ELIZA	Enzyme-linked immunosorbent assay
PZA	Pyrazinamide
RIF	Rifampicin
EMB	Ethambutol
INH	Isoniazid
ETH	Ethionamide
RB	Rifabutin
sPLS-DA	Partial Least Squares Discrimination Analysis
FU	Follow-up
VL	Viral Load
BSA	Body Surface Area
MTC	Multiple testing correction
FC	Fold Change
PLHIV	People Living with HIV

SDC Sequential Drug Challenge

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List of tables:

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protein was present in the sample on average (i.e., how abundant were the proteins that were identified). In this case the proteins quantified were present across ~4.5 orders of magnitude

Figure S2: The TIC (total ion chromatograms) for each file show how consistently every sample ran. The produced data was very reproducible

Figure S3: The data normalization shows you what the distribution of the intensities of the precursors looked like before and after normalization. For a label free experiment, this is a means to determine that the loading was approximately equal per sample

Figure S4: The data points per peak indicates the median number data points that were used to calculate the AUC for each precursor.

Figure S5 (left): A Heatmap showing the gene intensity level for all acute patients

Figure S6 (right): A heatmap showing the gene intensity level for all samples included in the patient cohort

Figure S7: Box and Whiskers diagram which show the significantly up/downregulated proteins in the analysis

— **Abstract**

Introduction: A greater incidence of severe cutaneous adverse drug reaction (SCAR) such as Drug Reaction with Eosinophilia Systemic Symptoms (DRESS) occur among HIV-infected patients. We sought to characterize the immunoproteomic profile of DRESS to first-line TB drugs in HIV infected cases. We hypothesize that differentially regulated proteins would be found interindividually and with disease evolution that may provide a mechanistic understanding of DRESS underlying pathways.

Participants and Methods: HIV cases with DRESS (probable or definite) and confirmed reactions to either one or many first-line anti-TB (FLTB) drugs were chosen (n=8). Discovery High Performance Liquid Chromatography Mass Spectrometry (HPLC-MS) (data independent proteomic) analysis was carried out on plasma samples with downstream protein identification and quantification analysis done on Spectronaut ™. (https://biognosys.com/resources/spectronaut-15-expand-biological-insights-with-dia-proteomics/) Data and statistical analysis was carried out using Perseus and R programming language.

Results: Overall protein clustering suggested greater plasma proteome differences between individuals than intraindividually between DRESS disease states and drug rechallenge reactions. Acute phase proteins and immunoglobulin kappa variable chains dominate the serum proteome of acute DRESS in TB/HIV co-infected patients, with CRP having the highest fold change ($q = 2.5$) in acute versus early recovery samples. LRG and L-Selectin ($q < 0.05$, $FC > 1.5$), both proteins involved in maintenance and regulation of the immune system especially at endothelia, were found to be upregulated in acute compared to recovery samples. Multiple non-immune related proteins, linked to actin-binding and thus cell structure, morphology, and functioning, were also upregulated ($p < 0.05$, $FC > 1.5$) in acute samples (including (ACTB) Actin, cytoplasmic 1, (ACTC1) Actin, alpha cardiac muscle 1, (VCL) Viculin). (MPO) Positive drug reaction samples from single versus multiple drug reactors had differentially regulated immune proteins: myeloperoxidase ($p = 0.04$, $FC = 1.4$), CRP ($p = 0.04$, $FC = 5.2$) and (LYST) Lysosomal-trafficking regulator ($p = 0.04$, $FC > 2.7$), proteins linked to polymorphonuclear cell inflammation, acute phase response and antiviral lymphocyte responses respectively.

Conclusion: Acute phase proteins and non-discriminatory hypergammaglobulinemia dominated the plasma proteome of HIV/TB co-infection and DRESS to FLTD, with drivers including TB disease burden and acute DRESS being hard to discriminate. A few differentially regulated immune proteins, particularly when comparing single versus multiple drug reactors, highlight roles for polymorphonuclear cell inflammation, and antiviral lymphocyte responses beyond the exaggerate innate acute phase response. Further work with increased sample size is warranted to confirm these preliminary findings.

Literature Review

Introduction

Drug hypersensitivity, and adverse drug reactions, result in significant patient morbidity and represents a large burden for health care system and drug development(2). Treatment regimens can become severely compromised due to the presence of drug hypersensitivities that necessitate the use of potentially less effective regimens (2). Persons with HIV are a particularly vulnerable population in the context of severe, treatment limiting ADRs, with an estimated 2-100 fold increased risk of developing immune-mediated ADRs (3). They are also patients frequently with life-threatening infections that require multi-drug, prolonged regimens. It is therefore essential that strategies to prevent, understand and predict IM-ADRs in this patient population be developed.

IM-ADR, CADR and SCAR

Adverse drug reactions are any untoward medication effect experienced at normal therapeutic doses of the drug(4). ADRs can be characterized into type A and type B. Type A ADRs may result from ‘on target’ effects in which the drug action is predictable(5). In contrast, type B ADRs are ‘off target’ effects which are part of a heterogenous group of reactions in which there are various clinical manifestations and underlying mechanisms. Type B ADRs are commonly immune-mediated and result in a range of clinical manifestations. Immune-mediated ADRs (IM-ADRs) are reactions which involve primary immune cells including T-cell (delayed hypersensitivity, Gell-Coombs type IV) and B-cell (antibody mediated, Gell-Coombs type I-III) mediated reactions (6). **Figure 1** shows the proposed high-level classification of on-target and off-target reactions, with further classification of immune-mediated and non-immune mediated off target reactions. Certain off-target ADRs are directly immune-mediated and are associated with immunological memory (drug hypersensitivity) (6). ADRs may differ between individuals due to acquired and genetic host factors. However, it is proposed that the intensity of the interaction between the drug and the target may be the result of the dose and duration of treatment. In the figure, immunologically mediated drug hypersensitivity comprises of antibody-mediated and T-cell mediated off-target ADRs(5).

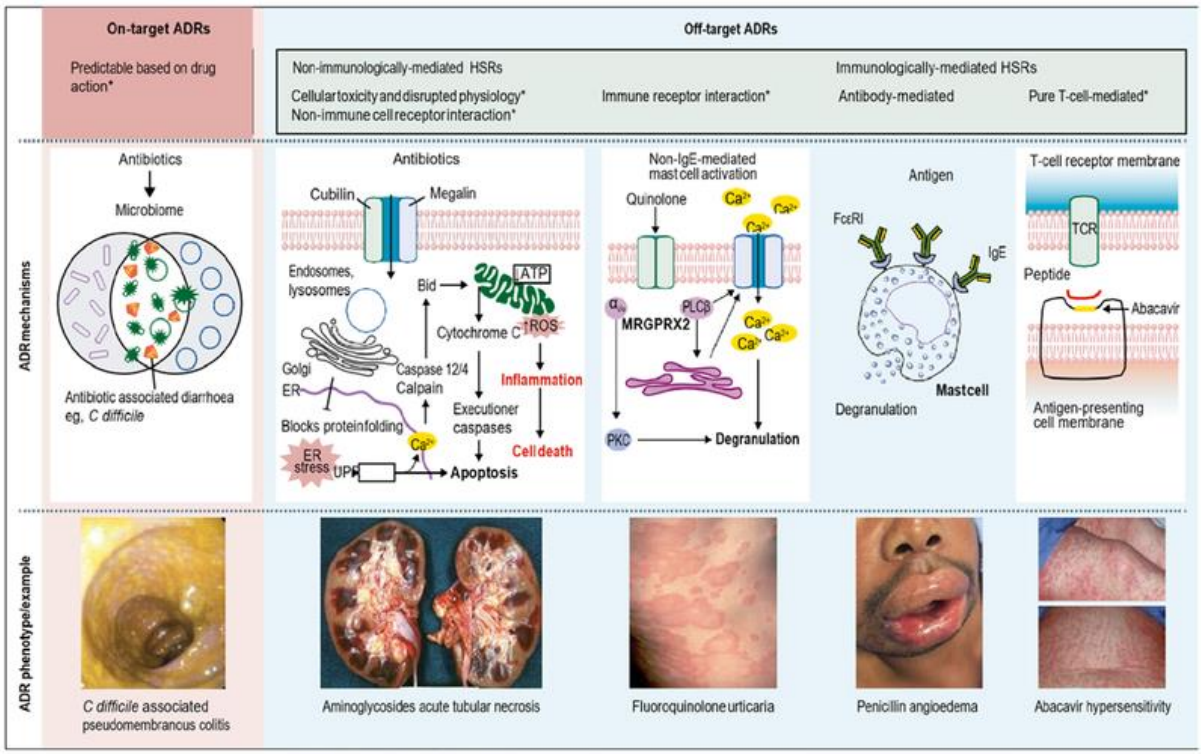


***Figure 1.1*: Classification of on-target and off-target ADRS Pink panel illustrates an example of an on-target ADR.** Blue panel (left) illustrates non-immunologically-mediated off-target effects: direct cellular toxicity or disruption of normal physiology, interaction with non-immune receptors, and interaction with immune receptors (e.g., non-IgE-mediated mast-cell activation via G-protein coupled receptors). Blue panel (right) shows immunologically mediated adaptive immune responses (antibody-mediated [e.g., IgE] immediate reactions or T-cell-mediated delayed reactions). Predisposition to both on-target and off-target reactions is driven by genetic variation, but also ecological factors that can vary over the course of an individual's lifetime. ADR=adverse drug reaction. Bid=BH3 interacting-domain death. C difficile=Clostridioides difficile. ER=endoplasmic reticulum. FcεR1=high-affinity IgE receptor. HSR=hypersensitivity reaction. MRGPRX2=MAS-related G-protein coupled receptor member X2. PKC=protein kinase C. PLCβ=phospholipase C β. ROS=reactive oxygen species. TCR=T cell receptor. UPR=unfolded protein response. *Dose-dependent. Figure adapted from *Peter et al (7)*.

The term IM-ADR encompasses all ADRs with an immune aetiology. Thus, the full range from type I – IV drug hypersensitivity reactions are included in IM-ADRs. Gell and Coombs categorizes hypersensitivity reactions into four main subtypes; Type I drug hypersensitivity is immediate or IgE mediated, type II is cytotoxic of IgG mediated, Type III is IgG/IgM immune complex mediated and lastly, type IV is delayed-type hypersensitivity or T-cell mediated

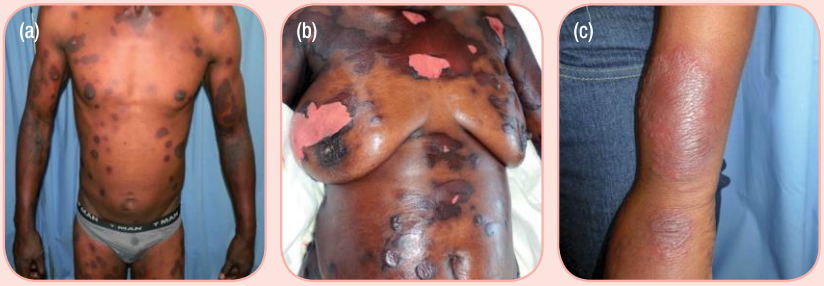
reactions (5). In HIV, the increased burden is in type IV delayed hypersensitivity or off-target reactions (5).

Cutaneous Adverse Drug Reactions (CADRs) are the commonest IM-ADR, and being Severe Cutaneous Adverse Drug Reactions (SCARs) are some of the most severe and life-threatening. CADRs are frequent, not life threatening and involve drug induced changes to the skin and mucous membranes underlying the skin (8). Although SCARs are less frequent, these reactions are potentially lethal. Globally, the most common SCAR phenotypes are drug reaction eosinophilia and systemic symptoms (DRESS), Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN). In South Africa, these adverse drug reactions occur with prevalence of 1/10 000 and 1.2-6/1 000 000, upon drug exposure, respectively (7). Immune-mediated adverse drug reactions occur up to 100 times more commonly in HIV infected people (9). With the common occurrence of an HIV and TB comorbidity, it is found that approximately 10% of HIV and TB coinfected patients are required to stop the use of offending drugs in multidrug resistant tuberculosis regimens due to ADRs (9).

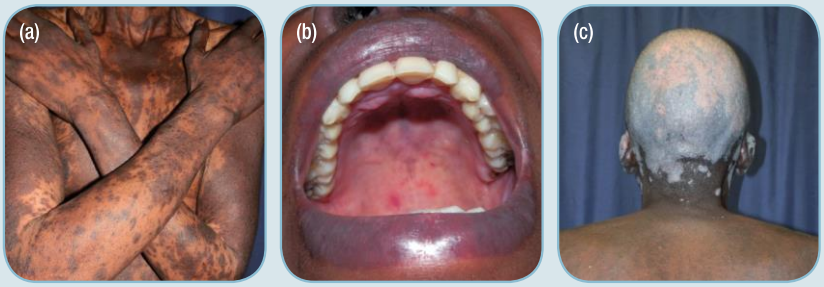
- **Major SCAR phenotypes – DRESS and SJS/TEN**

Clinical phenotypes of SCAR affected patients in HIV and TB endemic settings are shown in **Figure 1.2**. The commonest two SCAR phenotypes (>80% of all hospitalised cases) are DRESS and the spectrum of epidermal necrolysis, including SJS/TEN (10). Drug reaction eosinophilia and systemic syndrome, otherwise known as DRESS, is a multi-organ inflammatory response that typically occurs between 2- and 6-weeks post drug introduction. This syndrome is clinically characterized by a fever, varying cutaneous eruptions, widespread severe skin lesions, systemic involvement (particularly hepatic and renal dysfunction) and eosinophilia. With a mortality rate between 5-10%, patients diagnosed with DRESS require prolonged hospitalization (11). SJS/TEN are variants of the same condition in which the skin and mucous membranes are severely affected. This adverse drug reaction is an acute, rare and potentially fatal skin reaction in which there is a large scale epidermal death as a result of apoptosis, as noted in **Figure 1.2 (12)**. Although initial symptoms of this drug reaction are that of an upper respiratory tract infection, it soon manifests into epidermal detachments and haemorrhagic erosions of the mucous membranes. While SJS and TEN are considered a part of a single disease spectrum, they can be separated by degrees of skin detachment. SJS

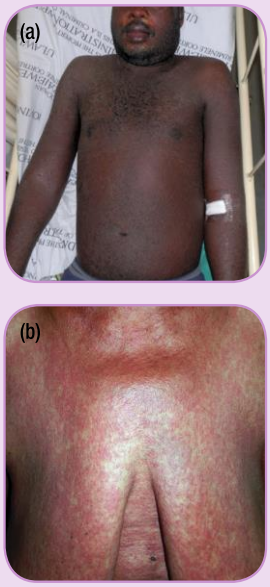
affects 10% of body surface area, while TEN affects up to 30% of the body surface area. The term SJS/TEN are for cases that range between 10-30% BSA affected (13).



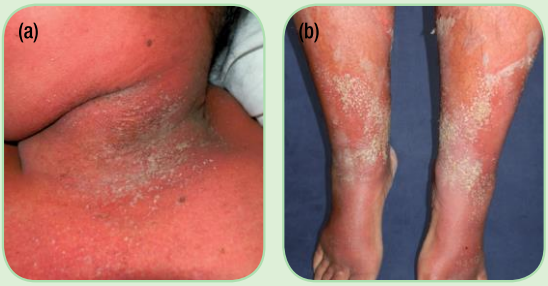
Fixed drug eruption *(a) Extensive disease showing pigmented macules, some with blistering (b) bullous variant resembling SJS and (c) acute fixed drug eruption showing indurated oedematous plaques.*



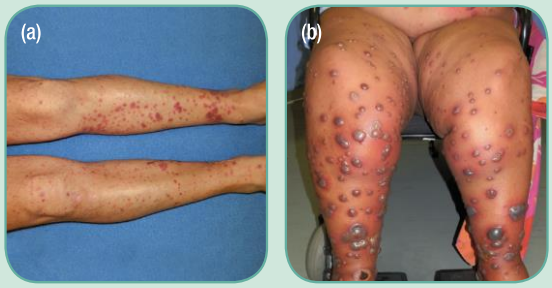
Lichenoid drug reaction (LDR) *showing (a) pigmented macules (b) violaceous erythema of the lips and (c) recurrence on re-exposure to the same drug. The depigmented area represents original sequelae of LDR and the violaceous areas developed on re-exposure to the drug.*



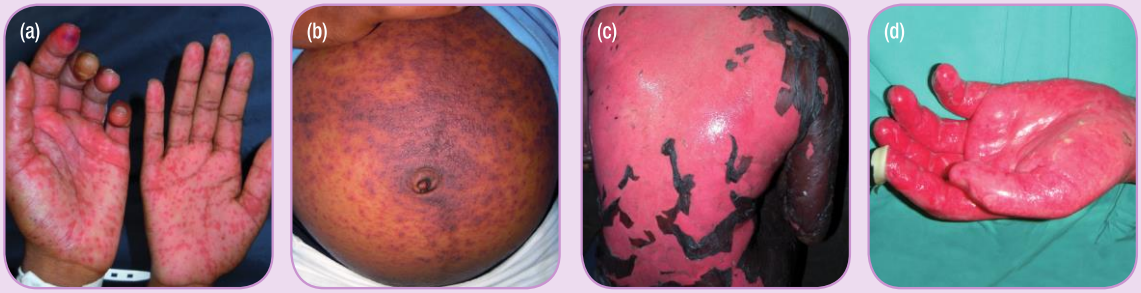
Drug reaction with eosinophilia and systemic symptoms (DRESS) *showing (a) exfoliative dermatitis with severe scaling in a background of erythema and (b) indurated erythematous macules and papules.*



Acute generalized exanthematous pustulosis (AGEP) *(a) classic flexural pustules (b) small pustules on a background of indurated erythema.*



Vasculitis *showing (a) palpable purpura on the lower legs and (b) a more severe variant showing blisters and ulceration.*



Stevens Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) *(a) focal tender erythematous macules on the palms in SJS (b) focal areas of early epidermal necrosis in a background of erythema in a pregnant woman with SJS (c and d) stripping of the epidermis on the back and palms in TEN.*

***Figure 1.2*: Montage of images to illustrate important features of different clinical phenotypes. Figure adapted from *Peter et al (7)*.**

- **Epidemiology of DRESS in African HIV and TB endemic setting**

Drug reaction with eosinophilia and systemic symptoms (DRESS) syndrome is one of the drug-induced severe cutaneous adverse reactions (SCARs) (11). It is a life-threatening disease with cutaneous presentation and internal organ involvement, and its mortality rate is about 10%. There is a well-known list of drugs associated with DRESS including: antiepileptics e.g. carbamazepine, allopurinol, and antibiotics e.g. cotrimoxazole and vancomycin (14). However, the epidemiology of DRESS in African HIV and TB settings is unique, driven largely by an increased incidence of DRESS amongst PLHIV and the prescription of antibiotics to treat TB and prevent opportunistic infections. Mild severity CADRs occur in 20-30% of all PLHIV starting antiretroviral therapy and anti-TB treatment (15). Fortunately, the majority of these are mild and can be easily treated through. In contrast, DRESS or SJS/TEN is less common, but is increased up to 100-fold in PLHIV (15). The causative agents of SCAR in HIV and TB endemic settings are distinct, with first-line anti-TB drugs and cotrimoxazole the commonest offending agents; our single tertiary centre sees 3-5 cases/month (16). All the first-line anti-TB drugs: Rifampicin, Isoniazid, Pyrazinamide and Ethambutol have been associated with DRESS in our setting (17). In all settings, these severe reactions result in prolonged hospitalisation, high cost of hospitalization and substantial long-term morbidity (13). However, in the context of HIV TB co-infection there are additional problems. TB the leading cause of death in PLHIV and with advance immunosuppression, the mortality of TB is very high (18). TB-HIV co-infected patients can ill afford treatment interruptions, which occur as a consequence of SCAR. Premature interruption can also worsen TB transmission of disease and accelerate the development of drug resistant TB, with significant public health consequences (19).

- **Clinical challenges of DRESS to anti-TB treatment in HIV context**

DRESS is a complex, multi-system disorder that is difficult to manage in any clinical setting. However, DRESS secondary to anti-TB drugs occurring in the context of advanced immunosuppression secondary to HIV poses several unique clinical challenges where translational research is urgently required. DRESS is a severe reaction which can result in prolonged hospitalisation, high cost of hospitalization and substantial long-term morbidity (16, 20).

» DRESS and HIV/TB co-infection

While DRESS has been described to be life-threatening illness, it is particularly problematic in the context of HIV TB co-infection (21). DRESS patients co-infected with HIV/TB results in them being at an incredibly high risk for opportunistic infections. Interestingly, some patients report tolerance to anti-TB drugs prior to the development of HIV but develop SCAR on subsequent exposures to first-line anti-TB drugs (FLTB) when co-infected with HIV, TB-HIV co-infected patients can ill afford treatment interruptions, which occur as a consequence of SCAR. Identifying the culprit drug is a major challenge in the context of 4-8 drug polypharmacy (22). Thus, any treatment interruptions are life-threatening and need to be minimised. Furthermore, TB is a complex disease to treat, requiring multiple drugs for prolonged period (23). Withdrawal of particular drugs e.g., Rifampicin, can threaten the likelihood of treatment success in the long term (24). This necessitates altered and prolonged regimens or use of more drugs with alternative toxicities. Every effort is made to ensure that patients utilise all possible effective first-line therapies.

» Polypharmacy in context of DRESS

Polypharmacy in the context of DRESS in HIV/TB makes identification of offending drug very challenging (22). Patients are commonly on four FLTD, in addition to cotrimoxazole which aids in reducing chances of opportunistic infection prophylaxis and sometime a FDC of three antiretrovirals. Internationally and in most situations, SCAR would be a contraindication to drug challenge or any re-exposure to a possible offending drug, however the high mortality rates and necessity for treatment has meant the need for drug rechallenge. Therefore, in an attempt to find an adequate treatment option for these patients, it is important to rapidly reintroduce all

effective drugs with minimal harm. In order to do this, identification of the causative drugs needs is required and there are three traditional methods for doing so:

- Skin and delayed intradermals
- Patch testing
- Sequential drug challenge

Consensus on performance of intradermal testing (IDT) involves injection of 0.02 to 0.05 mL of the highest nonirritating drug concentration and needle gauge with reagent applied bevel-up, to the volar forearm of skin (25). The definition of a positive delayed IDT has varied but in general is defined as an erythematous induration or swelling at the IDT injection site at 24, 48, and out to 72 hours if negative at 48 hours. By applying drug allergens in a soluble vehicle under occlusion on intact skin, patch testing (PT) aims to safely reproduce in the confined area of the test the original delayed IM-ADR (25). Patch testing involves the use of in the context of HIV and TB we have found both the yield and sensitivity of patch testing to be low and there have also been downstream systemic reactions to patch (lehloenya publications) Lastly, Sequential Drug Challenge (SDC) is used as the most common method for culprit drug identification. This method involves reintroducing each anti-TB medication sequentially until a reaction arises (26). As a reaction arises, the culprit drug is then identified. Multiple drug reactors have been noted in the SDC, with very different phenotypes of reactions which makes identification difficult and ambigious.

- **Considerations of Sequential drug rechallenge**

Early diagnosis and immediate withdrawal of the suspected drug is a key tenet of management of DRESS (27). The eruption is often urticaria-like and maculopapular, often generalizing into a severe exfoliative dermatitis or erythroderma (28). This phenotype has been studied in extensive detail and is contributable to the introduction of anti-TB medication. More specifically, the anti-TB medication commonly in question is the four first line TB medication including Isoniazid, Rifampicin, Ethambutol and Pyrazinamide.

In vivo drug provocation testing with offending drug(s) is the gold standard method for identifying causative drugs (29). This is particularly useful in the clinical setting where patients develop SCAR while receiving multiple drugs concurrently, which is the common occurrence in the context of HIV and TB co-infections. In most settings of SCAR, oral rechallenge is considered contra-indicated due to the risk of reoccurrence of severe symptoms following exposure to even a single dose of an offending drug (7). However, the need for effective anti-TB treatment, particularly in the context of HIV co-infection and increasing drug-resistance, necessitated a clinical imperative to ensure that the fewest possible drugs are eliminated from any anti-TB drug regimen.

» Rechallenge protocol

The common procedure for drug challenges includes a full-dose drug challenge followed by 1 to 2 hours of observation or a graded challenge including one-tenth to one-quarter of a dose followed either 30 to 60 minutes later or 3 to 7 days later by the full dose (25). For diagnostic purposes for delayed reactions, rechallenges involving greater than 2 steps have been avoided because of the concern that multistep multidose challenges may lead to desensitization. While this method is considered the gold standard for culprit drug identification, it does not come without its downfalls. Lower single-dose oral challenges that will adequately exclude IgE-mediated reactions (e.g., amoxicillin 250 mg) may not adequately exclude delayed T-cell-mediated reactions. In addition, several studies, particularly in the context of antibiotics, have suggested that single-dose challenges with the treatment dose of a drug will not exclude a delayed reaction and that multiple-day (3- to 10-day) challenges will pick up an additional 5% to 15% of reactions (25). Moreover, rechallenging patients may result in different phenotypes of reactions to both FLTD and second-line drugs (30).

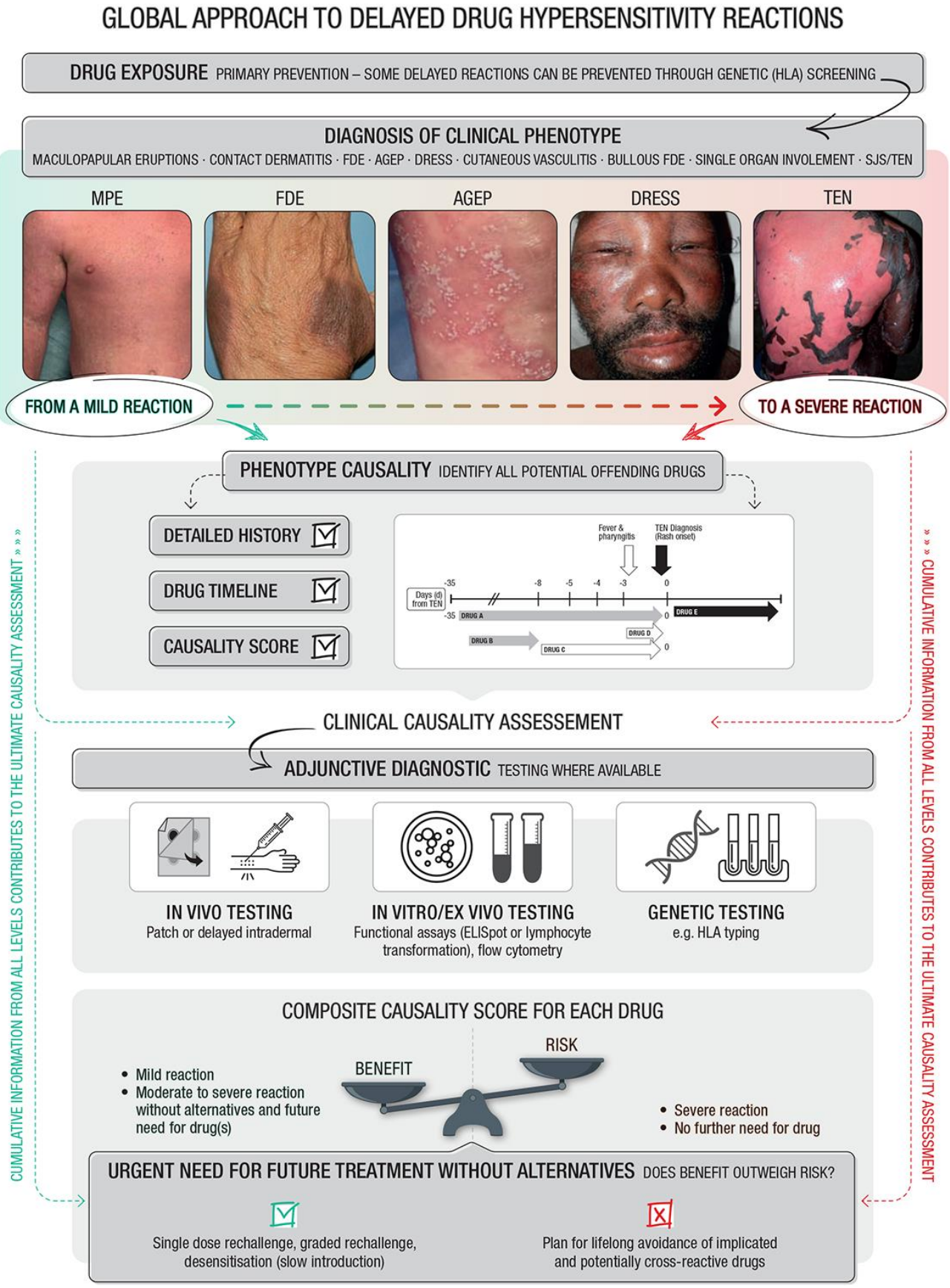


***Figure 1.3:** This figure outlines strategies to prioritize and obtain as feasible all levels of information required to diagnosis delayed immune-mediated adverse drug reactions*

(IM-ADRs), correctly identify offending drugs, and make decisions around future drug(s) avoidance or re-challenge. *Genetic testing e.g., HLA-typing, should be considered as a screening test where testing is available, where the number needed to treat to prevent a given IM-ADR is favorable and when time allows for testing to be done prior to initiation of therapy. Recognition of the clinical phenotype is the usual starting point in delayed hypersensitivity with stratification of mild to severe IM-ADRs; for severe IM-ADRs this often requires in-patient monitoring and adjunctive laboratory testing. A detailed history including drug exposure timelines allows consideration of all possible offending drugs, and causality assessments e.g., Naranjo should be performed for all possible offenders. The drug timeline shown for a patient who develops toxic epidermal necrolysis (TEN) in Figure 2 is key to identifying the most likely drug. For instance, in the timeline above, drug D and E can be ruled out as implicated as both are started after the onset of TEN symptoms. The latency period for drug A (35 days) is longer than average for SJS/TEN (4–28 weeks) making Drug C the most likely implicated drug associated with TEN. The particular offending drug; known diagnostic accuracy data in specific phenotype or population; and local availability and experience determines which adjunctive diagnostic testing should be applied. All preceding cumulative information is then weighed against the need for treatment and availability of effective alternative drugs, and depending on this risk benefit stratification, an informed decision can be made about the need for lifelong avoidance (implicated and cross-reactive drugs) or one of several strategies of drug re-exposure and a repeated attempt at treatment.*

As noted in Table 1, a number of patients have reactions to more than one drug (the multiple reactors) and the heterogeneity that can arise from sequential drug challenge.

Table 1.1: **Patients with FLTD SCAR reactions showing multiple drug hypersensitivity and diverse clinical phenotypes to fluoroquinolones and 2nd line anti-TB drugs**

Initial type of SCAR	Offending 1st line TB drug	CD4 cell count	Clinical reaction to FQ	Offending FT (or other possible drug)	Timing of reaction
DRESS	HE	191	Hepatitis	M	Delayed

DRESS	E	187	Anaphylactoid reaction, hypotension	M	Immediate
DRESS	RZ	153	Rash, hepatitis, oedema, eosinophilia	M, Et, T	Accelerated
DRESS	None	232	Itch, fever, oedema, hepatitis, headache	O, S	Accelerated
DRESS	R	401	N/V, oedema, fever, rash, hepatitis	M	Delayed
DRESS	H	145	Itch, rash, nausea	O	Delayed
DRESS	None	214	Itch, rash, fever, erythema	M	Accelerated
DRESS	H	457	Fever, rash, pruritus	M	Accelerated
DRESS	R	32	Eosinophilia, fever, diarrhoea	M	Delayed
DRESS	None	395	Fever, oedema, erythema, hepatitis	O, S	Delayed
DRESS	None	81	N/V, hypotension	M	Accelerated
DRESS	None	12	Fever, rash, eosinophilia, hepatitis	O, S, Et	Accelerated
DRESS	E	13	Fever, rash, eosinophilia, hepatitis	M	Delayed

DRESS	None	64	Erythema, rigors, N/V, oedema	O, S, Et	Immediate
SJS/TEN	Z	649	Fever, rash, eosinophilia, hepatitis	M, I, C, Am	Immediate
TNE	None	234	Painful hands and feet, blistering	O. S	Accelerated
SJS/TEN	H	1	Itch, oedema, fever, eosinophilia	O, S	Immediate
SJS	None	201	Anaphylactoid, hypotension	O, S, Et	Immediate

Abbreviations: ADME Absorption, Distribution, Metabolism and Elimination | ADR Adverse Drug Reaction | AGEP Acute Generalized Exanthematcus Pustolosis | AIN Acute Interstitial Nephritis | AR Anaphylactoid Reaction | ART Antiretroviral Therapy | CADR Cutaneous Adverse Drug Reaction | CNS Central Nervous System | CTCAE Common Terminology Criteria for Adverse Events | DILI Drug-induced Liver Injury | DPT Drug Provocation Testing (Drug Challenge or re-challenge) | DRESS Drug Rash with Eosinophilia and Systemic Symptoms | FLTD 1st line anti-TB drug | GSH Groote Schuur Hospital | HIV Immunodeficiency Virus | HLA Human Leucocyte Antigen (Major Histocompatibility Complex) | IDM Institute of Infectious Diseases and Molecular Medicine | IDT Intradermal Test | IM=ADR Immune-mediated Adverse Drug Reaction | IRIS Immune Reconstitution Inflammatory Syndrome | LDE Lichenoid Drug Eruption | N/V Nausea and Vomiting | NVP Nevirapine | TB Tuberculosis | TCR T-cell Receptor | TLR Treatment Limiting Reaction | Individual drug: Am Amikacin, C Capreomycin, E Ethambutol, Et Ethionamide, H Isoniazid, L Linezolid, M Moxifloxacin, O Ofloxacin, R Rifampicin, S Streptomycin, T Terizidone, Z Pyrazinamide

Groote Schuur Hospital (GSH) dermatology and drug allergy clinic have, over the last several years, gained extensive experience and insight into SCAR on re-challenging patients with first line TB therapy. They have observed multiple different symptoms and varied time responses of drug provocation testing reactions, including both immediate and delayed type reactions. These reactions range from mild to potentially life threatening, the latter including SJS/TEN and DRESS. Furthermore, patients who have had a cutaneous adverse drug reaction appear

to be more susceptible to reacting to other drugs (such as fluoroquinolones) which are chemically distinct from the original culprit drug; and notably rechallenge reactions (RR) can produce varied clinical manifestations, often distinct from the initial SCAR phenotype (8) (see table 1). This is particularly true for HIV infected patients. The clinical spectrum of rechallenge reactions includes immediate (<1 hour to symptoms) with anaphylactoid type symptoms and delayed (>6 hours) with more typical features such as rash worsening. While the rechallenge reactions are evident in HIV infected DRESS patients, flare-up reactions can also occur in HIV unaffected patients diagnosed with DRESS (31). Flare-up reactions are late manifestations or more specifically relapses of drug-induced T-cell-mediated hypersensitivity reactions, in particular DRESS. While these flare-up reactions are often attributable to the reactivation of virus, in particular herpes virus, these reactions can also be associated with the stimulation of preactivated T cells by drugs binding to the TCR and HLA (31).

» DRESS Flare-up reactions in context of SDC

DRESS flare-ups as a result of the anti-TB drug introductions is an area of research that has been studied on a molecular and cellular level however, on a proteomic level, it is less well defined. Flare-up reactions are late manifestations or more specifically relapses of drug-induced T-cell-mediated hypersensitivity reactions, in particular DRESS (32, 33). These flare-up reactions may be attributable to the stimulation of preactivated T cells by drugs binding to TCR and HLA. They may also be related either to viral reactivations, or to administration of new drugs or to previously tolerated drugs after dose increase (33). *Picard et al* found that 25% of the DRESS patients had drug-related relapses (34). This further necessitates the urgency to understand these reactions better and more effective, long term treatment options. Biomarkers specific to these flare-ups are less well known. More specifically, biomarkers need to be identified for positive clinical reactions to distinguish non-specific flare-up reactions from a drug-specific response (with immunological memory).

Sequential drug challenge is an opportunity to better understand the immunopathogenesis and important immune networks involved in these DRESS flare-up reactions. While cellular responses have been studied in our laboratory, the soluble markers and immunoproteome have yet to be investigated, therefore this study will look into these two aspects in drug rechallenge samples in order to further understand the molecular causations of these reactions.

- **EiSpot data for in vitro identification of drug-specific T-cells**

While the rechallenge diagnosis is considered the gold standard it is unclear, given the diverse phenotypes of the reactions and the fact that patients have multiple reactions whether the reactions occurring represent “true” hypersensitivity reactions to specific pharmacological agents or transient, non-specific reactions that could be treated through. We have optimised the EiSpot to aid in the in vitro identification of drug-specific T-cells to FLTD. The EliSpot technique was initially developed to quantify secretion and activation of T-cell responses to specific viral peptides (25). For T-cell-mediated drug hypersensitivity, the assay has been adapted to quantify secretion and activation of responses to T cells stimulated by varying concentrations of suspected implicated drugs or metabolites. The number of spot-forming-units or spot-forming cells that release cytokine markers or cytolytic molecules, such as IFN-g and granzyme B (GrB), are quantified after the patient’s PBMCs are stimulated with pharmacologically relevant concentrations of the suspected drug(s) (25, 35).

In our laboratory, researchers have investigated the cellular response in the peripheral blood mononuclear cells to *in vivo* challenge. Figure 4 shows that there are higher levels of rifampicin-specific and Isoniazid-specific T-cell responses in the post-positive drug rechallenge sample. Our laboratory has since identified some positive reactions, particularly to Rifampicin, which is then enhanced by the *in vivo* drug rechallenge.

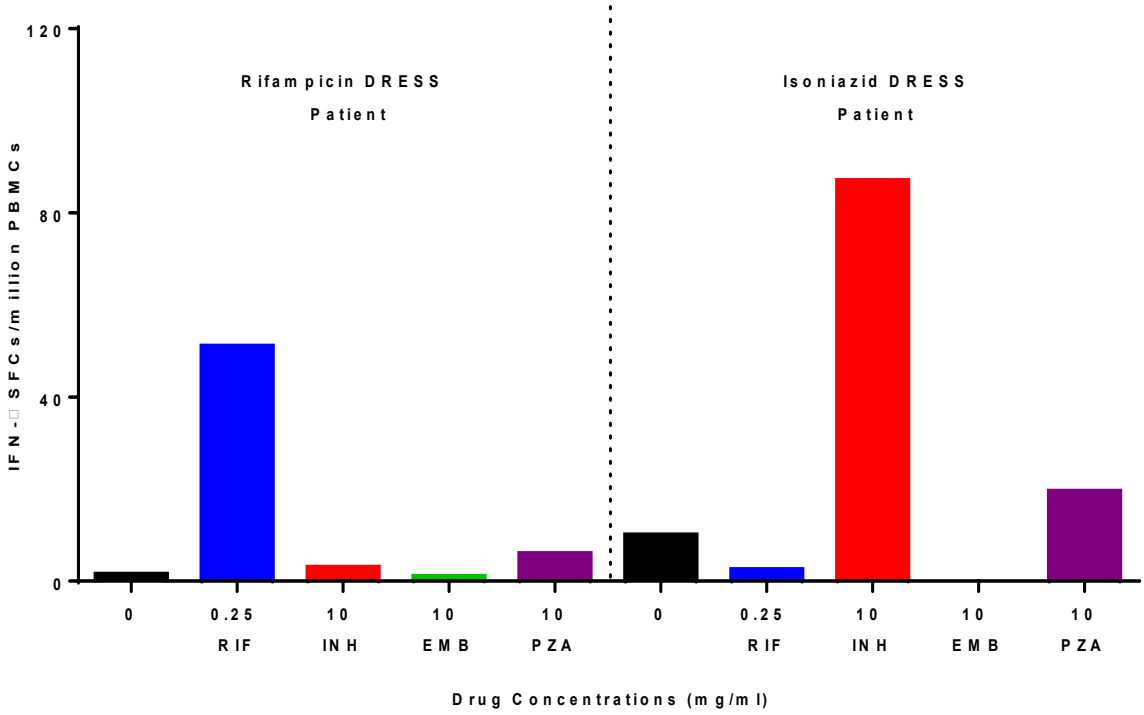


Figure 1.4: **An ELISpot assay to detect drug specific interferon gamma (IFN-γ) producing cells in DRESS patients after stimulation with first line anti-tuberculosis drugs.** PBMCs collected during drug provocation testing from two HIV infected patients with DRESS to rifampicin and isoniazid were stimulated in vitro with validated drug concentrations for 18hr. Each sport represented one cell that secreted IFN-γ and responses are represented as spot forming cells (SFC) per million PBMCs. RIF, Rifampicin, INH, Isoniazid, EMB, Ethambutol, PZA, Pyrazinamide, PBMCs, peripheral blood mononuclear cells, DRESS, drug reaction with eosinophilia and systemic systems.

- **Immunopathogenesis of SCAR, focus on DRESS**

» **SCAR pathogenesis**

The immunopathogenesis of SCAR is incompletely understood; the major mechanism is the critical role of effector T-cells that are highlighted in Figure 1-2. DRESS is thought to be mediated by both CD8+ and CD4+ T cells (36). It is characterized by an increased lymphatic infiltrate of T cells and eosinophils into the dermis, and increased release of inflammatory cytokines including TNF-α and IFN-γ. In SJS/TEN, the drug likely interacts with the human

leukocyte antigen protein (HLA) on keratinocytes that act as antigen presenting cells (APCs) to activate drug specific CD8+ cytotoxic T cells. This results in accumulation of these CD8+ T cells within epidermal blisters, releasing perforin and granzyme B that kill keratinocytes (7, 37). CD8+ T cells, NK cells, and NKT cells are also triggered to secrete granulysin, an important cytotoxic mediator in SJS/TEN which induces keratinocyte death without the need for cell contact and is correlated with severity of disease.

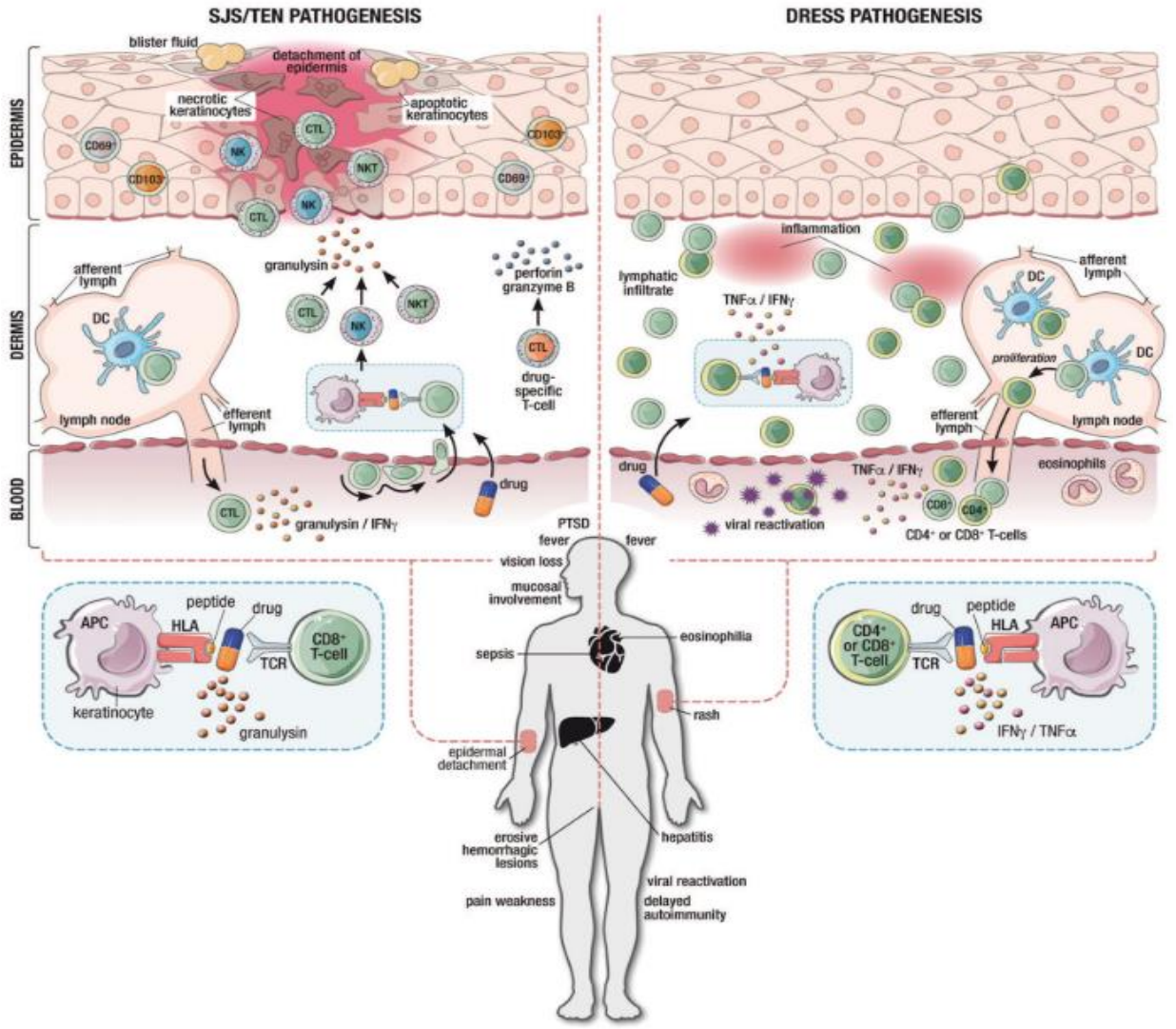


Figure 1.5: **The pathogenesis of SJS/TEN and DRESS. Figure adapted from *Peter et al [5]*.**

» HLA associations

Class I HLA restriction is necessary but not sufficient for SCAR, with many risk alleles carrying 100% negative predictive values, but low positive predictive value and why some 10 tolerant patients carrying the risk allele will not develop SCAR, indicates that additional determining factors must be present to develop these reactions. The same HLA risk allele for a particular drug can also results in different clinical phenotypes, for instance B*58:01 is associated with both allopurinol-induced DRESS and SJS/TEN and HLA-A*31:01 with carbamazepine-induced DRESS and SJS/TEN (38, 39). The factors that determine the phenotype of SCAR in such cases are unknown. Cross reactive expanded effector memory T-cell clones are suggested as an important additional factor in SCAR development. Several of the offending drugs implicated in SCAR amongst patients living with HIV have not had a clear HLA risk allele or dominant clonal TCR identified and therefore, it can be noted that there are multiple key factors required to distinguish tolerance from hypersensitivity. A study performed to investigate the association of DRESS syndrome to specific HLA alleles found the following; a CD8 T-cell–dependent hypersensitivity reaction during treatment with abacavir, an anti-HIV medication, is strongly associated with the presence of the HLA-B*57:01 allele (40). An initial, important clue to the HLA association with DRESS was the strikingly lower incidence of abacavir hypersensitivity in persons of African ethnicity, who have a much lower prevalence of HLA-B*57:01 than white persons (38, 41).

» T-cell response

Immune-mediated adverse drug reactions (IM-ADRs) are a large source of morbidity, mortality, and burden on health care systems; however, these reactions are often preventable. IM-ADRs encompass phenotypically distinct diagnoses that comprise of both B-cell and T-cell mediated reactions. It is strongly suggested that SCARs, specifically DRESS and SJS/TEN, are T-cell mediated drug reactions (42). Antigen presenting cells (APC) present antigens via the major histocompatibility complex (MHC), also known as the human leukocyte antigen (HLA) complex. T-cells then bind to these peptide antigens present on APC cells via a unique T-cell receptor present on the T-cell surface. Antigens presented via class I HLA complex are recognized by CD8+ T-cells while CD4+ cells recognise antigens presented via class II HLA complex. Engagement of the TCR and antigen-HLA complex results in cell clonal proliferation and differentiation into effector and memory phenotypes (43). Activation of distinct types of drug-specific T-cell responses e.g., TH1 or TH2 or IL-8/GM-CSF are thought to lead to specific clinical phenotypes of SCAR e.g., SJS/TEN versus DRESS. All the major T-cell subsets such as cytotoxic T lymphocytes (CTLs), T-helper cells including Th1 and Th2 and regulatory T-cells (T_{REG}) are thought to be involved in pathogenesis, but their relative contributions to pathogenesis, as well as involvement of other immune cells still remain to be elucidated, especially in the context of HIV related immune dysregulation (44). The immunopathogenic profile of this DRESS is mediated by CD4+, CD8+ T-cells and, more specifically, Interferon (IFN)-γ, Interleukin 5 (IL-5), Granzyme B and tumour necrosis factor (TNF)-α as Immunohistochemistry data has shown (45).

» **Viral reactivation**

The role of viruses in the pathogenesis of DRESS is unclear however, certain mechanisms have been proposed; a) Viral reactivation may be provoked by a cytokine storm secondary to an immune response against the drug (26); b) DRESS is a consequence of a strong immune response against an early viral reactivation (46). CD4+ and CD8+ drug-specific T cells proliferate after encountering the drug, but also anti-viral specific T cell can be cross-activated by drugs. The development of a TRM population which persists at the site of the antigen encounter at the skin. These virus-specific effector memory T-cells can cross react with drug-induced self-peptides presented by HLA risk allele, resulting in drug hypersensitivity reactions (41, 42). The role HIV-specific T-cells, particularly TRM, play in mediating SCAR is unknown, although HIV-specific T-cell clones have been demonstrated to react in vitro against abacavir-altered peptide repertoire (47), suggesting that HIV-specific memory T-cells can contribute to hypersensitivity reactions through heterologous immunity (48). In conclusion, the most

common hypothesis is that the immunologic response to drugs induces a boost viral reactivation, consequently T lymphocytes and monocytes/macrophages release viruses that represents as an early marker of stimulation of these cells, rather than the triggering event in the pathogenesis of DRESS (26).

» Soluble and other factors in DRESS pathogenesis

› JAK/STAT pathways

Progress in single-cell RNA sequencing (scRNAseq) provides an opportunity to delve deeper into human disease pathophysiology, particularly in diseases lacking animal models, such as DRESS. Performance of scRNAseq on skin from a DRESS case highlighted the JAK-STAT signalling pathway and further identified that central memory CD4+ T cells were enriched with HHV6b DNA. Intervention via tofacitinib enabled disease control and tapering of other immunosuppressive agents. Furthermore, tofacitinib, as well as anti-viral agents, suppressed culprit-induced T cell proliferation *in vitro*, identifying the JAK-STAT pathway and herpesviruses as potential therapeutic targets in DRESS (49). Cytokines involved in the pathogenesis of DRESS, including IL-5, IL-13, TNF-α and IFN-γ, are signalled and activated through the JAK-STAT pathway furthering the evidence that the JAK-STAT pathway is involved in progressing DRESS pathogenesis. The JAK-STAT pathway is comprised of three main proteins; cell-surface receptors, Janus kinases (JAK) and signal transducer and activator of transcription proteins (STAT) (50). These three proteins are proposed to be expressed in differing levels throughout disease progression and may be attributable to differing disease severity in patients. It is therefore critical to investigate this pathway as a potential therapeutic target or biomarker of DRESS.

› Immunological mechanism of virus reactivation

Despite the strong association between HHV-6 and DRESS, the immunological mechanism underlying HHV-6 reactivation remains unknown. HHV-6 is known to exhibit selective tropism for CD4 T cells and to latently infect monocytes/macrophages (51, 52). Recently, it was found that CD134 is preferentially expressed on CD4 T cells in the acute stage of DRESS, while this was not observed in other types of drug eruptions (53). CD134, which is also called OX40, is a member of the tumour necrosis factor (TNF) receptor superfamily and is a well-characterized

co-stimulatory receptor (54). It was recently identified as a cellular receptor for HHV-6 (55). It is speculated that the upregulated expression of CD134 may contribute to the entry of HHV-6 into CD4 T cells in the acute stage of DRESS. Furthermore, it has been found that the number of circulating monomyeloid precursors with the CD11b+CD13+CD14−CD16 high phenotype increased in the early stages of cases of DIHS harbouring the HHV-6 antigen and that these cells were able to transmit HHV-6 to skin-infiltrating CD4 T cells (56, 57).

› Fas-FasL Interaction

Fas ligand (FasL) is part the tumour necrosis factor (TNF) family. As Fas and FasL binds, the pathways that follow play a role in regulating the immune system. The Fas-FasL interaction is also involved in the apoptosis of epidermal cells in patients with drug hypersensitivity. During Fas–FasL interaction, the Fas-associated death domain protein (FADD) is signalled which interacts and binds to the Fas–FasL complex. This death domain protein (FADD) signals procaspase 8, bringing multiple copies of procaspase 8 together, which in turn autoactivate to become caspase 8, triggering the caspase cascade. This cascade activates the intracellular DNA degradation pathway (58). Viard et al. proposed that a suicidal interaction between Fas and FasL, which are both expressed by keratinocytes, leads to the extensive necrosis of epidermal cells in individuals with DRESS and SJS/TEN (59).

› Perforin/Granzyme B

Granzymes are serine proteases released by cytoplasmic granules resulting in programmed cell death/apoptosis in the target cells. As drug-specific cytotoxic T lymphocytes (CTL) and NK cells are activated, they produce perforin, which can then bind to and create a channel through the cell membrane. This channel allows for the entry of granzyme B into the target cells signalling and activating the caspase cascade and the resulting apoptosis (60). Delayed reactions to drugs have shown that increasing levels of perforin and granzyme B are related to the disease severity of drug hypersensitivity (58).

› Granulysin.

Cytotoxic T cells and Natural Killer cells release a cytolytic protein called Granulysin. This protein create holes in the cell membranes to destroy target cells. Yang et al demonstrated that granulysin secretion is strongly expressed in patients with drug-

induced DRESS, and SJS/TEN which may be leading to apoptosis of target cells and increased drug hypersensitivity (61, 62).

› TNF-α, IFN-γ, TARC, IL-15, and Other Cytokines/Chemokines in SJS/TEN and DRESS/DIHS.

TNF-*α* is a major proinflammatory cytokine and is produced by macrophages, T lymphocytes, NK cells, neutrophils, mast cells, and eosinophils. It regulates immune responses through the induction of cell apoptosis, activation, differentiation, and inflammation (63). TNF-*α* was highly expressed and suggested to play a significant role in the extensive necrosis of skin lesions of SCAR patients (64). IFN-*γ* is critical for both innate and adaptive immunity against viral and bacterial infection, and it is predominantly produced by CD4+ T helper cells, CD8+ CTL, and NK cells. IFN-*γ* was found to be increased in the skin tissue, blister cells, and plasma of SJS/TEN and DRESS patients (58, 65). DRESS is characterized by leukocytosis with atypical lymphocytosis or eosinophilia [149]. Serum thymus and activation-regulated chemokine (TARC) was recognized as a potential biomarker for early onset of the disease and an indicator of disease activity in DRESS (66, 67). Compared to patients with SJS/TEN, the TARC levels in patients with DRESS are significantly higher during the acute phase and are correlated with skin eruptions (67). In addition, other cytokines and chemokine receptors, including IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-18, CCR3, CXCR3, CXCR4, and CCR10, have been found to be upregulated in the skin lesions, blister fluids, PBMC, or plasma of drug hypersensitivity patients and play a role in immune regulation of drug hypersensitivity (37, 58, 65, 68, 69)

› Syndrome-Specific Effector Cells.

Immunological changes of DRESS are characterized by the increase of atypical lymphocytes or eosinophils (70, 71). Eosinophilia can be observed in 60–95% of DRESS patients at the early stage of the illness (70). Most of DRESS patients had increased numbers of CD4+ T cells in the acute stage, which was associated with the severity of clinical symptoms, such as the extent of skin rash and reactivations of virus (72). In addition, Tregs play important roles in DRESS pathogenesis. Dramatic expansions of functional Tregs are found in the acute stage of DRESS (73). It is hypothesized that CD4+FoxP3+ T cells that home to skin serve to limit the severity of acute disease by regulating the cytotoxic effector T cell responses. However, Treg

responses are eventually exhausted and this might contribute to ongoing viral replication and intermittent recurrence of clinical symptoms (73).

» HIV factors linked to SCAR pathogenesis

The relation of HIV infection and SCAR pathogenesis is thought to be multifactorial, an interplay between metabolic, immunologic, host and viral factors. HIV is known to result in an increased susceptibility to drug reactions and accelerated SCAR pathogenesis. The pathways and factors behind this increased susceptibility are noted in **figure 1.6** below. Here, the pre-HIV genetic risk, and the dynamics of immunologic, metabolic (both disease-related and drug-related), pharmacologic and infective factors along the course of HIV infection are depicted (74). Persistent damage of HIV-infected cells, immune dysregulation, increased oxidative stress, and depletion of immunoregulatory cells are assumed exacerbate the cascade of immune responses, cytokine release and hypersensitivity reactions

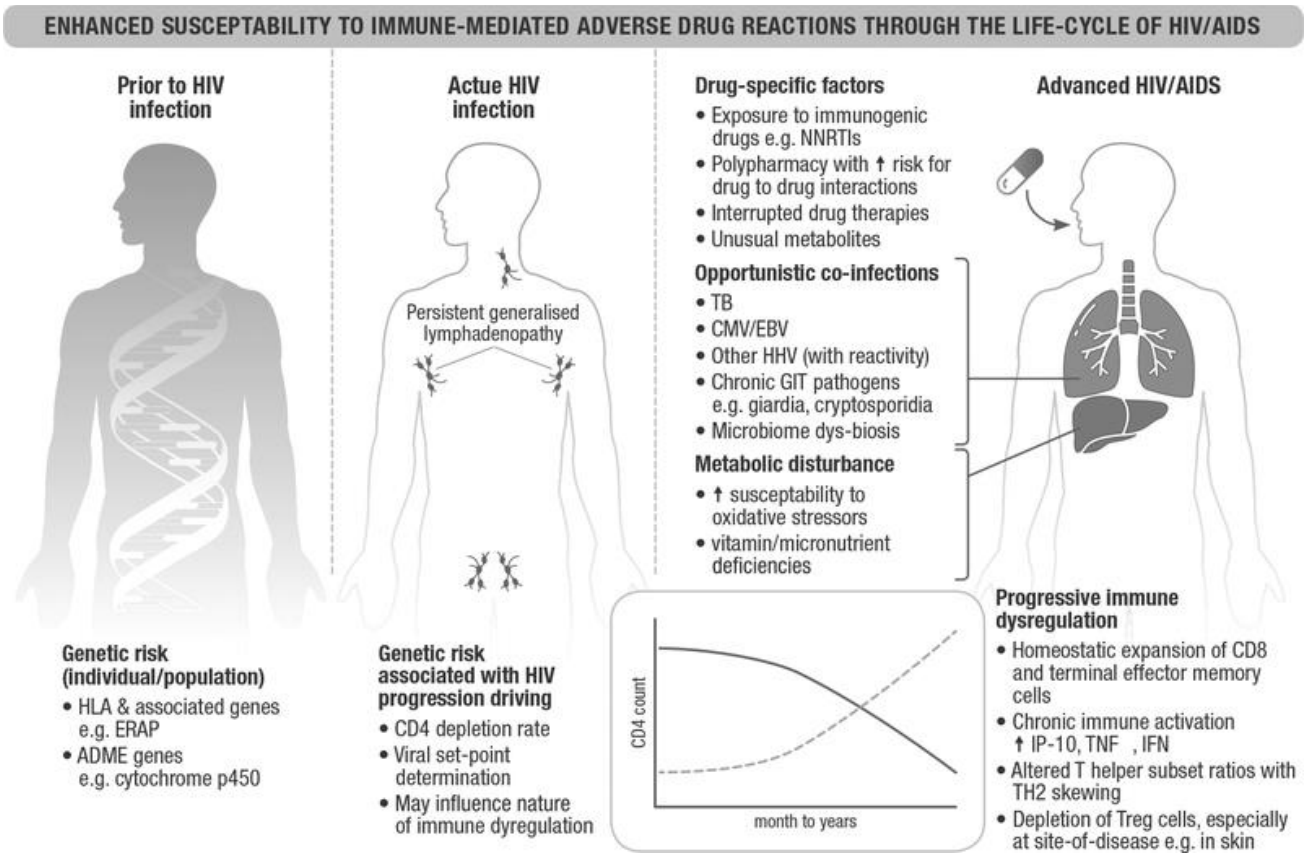


Figure 1.6: Enhanced susceptibility to immune-mediated adverse drug reactions through the life cycle of HIV/AIDS. *Several potential mechanisms may explain the increased*

susceptibility to IM-ADRs through HIV/AIDS life cycle. Prior to infection, individuals or populations may carry specific HLA or other pharmacogenomic risk alleles to particular drugs. Host factors determining HIV control may influence the dynamics of immune dysregulation. Upon infection and as disease progresses, CD4+ T cells are depleted, with homeostatic expansion of CD8+ T cells, particularly functional memory CD8+ T cells. Marked immune hyperactivation characterized by excessive levels of inflammatory cytokines, such as type I interferons (IFN-α) and suppressive cytokines, IL-10 and changes in the expression levels of IFN-γ induced chemokines (IP-10). Another influence includes altered immunoregulatory pathways, which results in the lack of counterbalance against cytotoxic immune responses. Reactivation of comorbid herpes viral infections, such as CMV, EBV, of HHV6 and HHV8 may occur during worsening immunosuppression; viral reactivation has a known association with DRESS. HIV can alter redox balance and potentially increase the production and accumulation of toxic reactive metabolites. Overall, during chronic infection, uncontrolled immune activation, loss of CD4+ T cells and Tregs and unopposed CD8+ cytotoxic T-cell responses likely promotes the enhanced susceptibility to IM-ADRs in HIV/AIDS patients. The precise balance of these signals may determine the severity of these reactions in different stages of infection. ADME, absorption, distribution, metabolism, and elimination; CMV, cytomegalovirus; DRESS, drug reaction with eosinophilia and systemic symptoms; EBV, Epstein–Barr virus; HHVs, human herpes viruses; IFN-α, interferon-alpha; IFN-γ, interferon-gamma; IM-ADR, immune-mediated adverse drug reaction; IP-10, interferon gamma-induced protein 10; TB, tuberculosis; TH2, T-helper 2; TNF, tumour necrosis factor; Treg, regulatory T cells.

Figure adapted from Peter et al, 2017

HIV infected individuals are found to be at a greater risk for developing IM-ADRs for varying reasons. Firstly, genetic predisposition (particularly HLA risk) is important for several delayed IM-ADRs and has been studied for a number of drugs relevant to HIV, including the antiretrovirals (75). As previously discussed, these genetic risk factors are critical determinants of certain IM-ADRs prior to HIV infection. In fact, even in IM-ADRs with a clear HLA risk allele, the positive predictive values are low (<10%), meaning other additional factors are necessary for a reaction.

Secondly, the progression of HIV infection is characterized by loss of CD4 T cells; expansion of CD8 T cells; and chronic immune activation. Immune activation is associated with an increase in the production of IP-10 and MIG (chemokines induced by interferon-*γ*), TNF-*α*, IL-6, IFN-*α* and IL-10 (76, 77). This immune activation and increase in interferon-gamma levels may result in an increased drug presentation through the upregulation of HLA molecules and other co-stimulatory molecules on antigen presenting cells (APCs). Furthermore, the loss of Tregs – a $CD4^+$ T-cell subset may aggravate the ability to counterbalance cytotoxic CD8 cells, and this has been suggested in the skin for SCAR (73, 78).

Lastly, HIV-infected individuals have an exacerbated vulnerability to oxidative stress, which has been thought to increase exposure to toxic drug metabolites and potentially lead to the onset of IM-ADRs. Glutathione, a cellular antioxidant responsible for detoxifying toxic reactive metabolites and subsequently preventing tissue and cell damage, is found to be depleted in HIV infected individuals (79). This brings to light the importance of increased oxidative stress as a risk factor for susceptibility to drug hypersensitivity reactions in HIV-infected patients (80).

» **Immunodysregulation and CD4+ depletion**

HIV infected individuals portray severe immune dysregulation and, as a consequence, are subject to multiple disorders. One of the major hallmarks of HIV infection is the progressive depletion of CD4+ T-cells (81). This loss of CD4+ T cell function and number is closely associated with impaired cellular immunity. Other T-cell subsets such as Th17 and T_{REG} cells which both function to regulate tissue inflammation are reported to be susceptible during HIV infection (82). HIV infection leads to a reduction in the functioning of Th17 cells in addition to alteration in T_{REG} immunosuppressive activity. Aberration in these cells' functioning causes a lack of proper maintenance of barrier integrity and suppression of cutaneous immune responses. Moreover, it is reported that a Th1 to Th2 cytokine profile switch is seen in HIV infected individuals (83). This is characterized by an increase in Th2 cytokines, namely IL-4 and IL-5, and a decrease in Th1 cytokines, namely IFN-γ. While HIV infected individuals are susceptible to adverse drug reactions due to the multidrug regimen, these individuals are also subject to the onset of skin disorders as a result of severe cutaneous immune dysregulation and progressive immunosuppression (84). These skin disorders include HIV-associated Psoriasis, Atopic Dermatitis and Papular pruritic eruption (PPE).

Susceptibility to drug hypersensitivity is evident in HIV infected individuals as a result of multiple factors including multiple comorbidities including infections, malignancies, metabolic and noncommunicable disease; polypharmacy with attendant drug–drug interactions and nonimmune ADRs, and under-resourced health systems in high-burden settings (39). IM-ADRs in persons living with HIV are victim to further problems including a predisposition to multiple drug hypersensitivity, inconsistent phenotyping, and very limited capacity for site-of-disease sampling, and no predictive or prognostic biomarkers (39). Another interesting link between HIV and DRESS pathogenesis is the CD4+ depletion. This CD+depletion has been speculated to play a role in disease progression in DRESS patients (85). Extensive research is required to delve deeper into these reactions in an attempt to

identify biomarkers in addition to having a clearer understanding of the immunopathogenesis of DRESS.

Antiretroviral therapy (ART) initiation in HIV infected individuals allows for the recovery of CD4+ T-cell numbers as well as a restoration of immune responses, thereby reducing the onset of opportunistic comorbidities and allowing for prolonged survival (86). However, following the initiation of ART, it is found that certain patients develop Immune Reconstitution Inflammatory Syndrome (IRIS) (87). IRIS occurs in the context of HIV and results in several systemic manifestations. Certain authors have drawn parallels between DRESS and IRIS. While the immunopathogenesis of IRIS remains relatively unknown, it is thought to be mediated an unbalanced reconstitution of regulatory and effector T cells and a major upregulation of innate cytokines (87). Due to this increase in cytokines seen in IRIS and DRESS, it brings to light the need for further research into the effect that the increase in cytokines has on the pathogenesis of DRESS.

» Oxidative stress pathways

HIV is the leading cause of death in many parts of the world and disproportionally apparent in areas of extreme poverty and socially disadvantaged communities (88). As a result, many HIV infected individuals suffer from food insecurities and malnutrition which may play a role in disease progression. Certain therapies including micronutrient replenishment could provide potential benefits in HIV disease. More specifically, focus must be placed on the micronutrient Selenium. Selenium is a micronutrient found in soil and functions in DNA oxidative damage repair, DNA synthesis and cellular signalling (89). It is reported that HIV patients are more susceptible to oxidative stress in which there is an overabundance of free radicals. These free radicals can damage cells and lead to inflammation. In order to control oxidative stress in the body, Glutathione participates in the detoxification of free radicals (79). Glutathione levels are found to be suboptimal in HIV infected individuals resulting in an increase in oxidative stress. This leads to DNA and cellular damage and decreased cell survival (79). Ultimately, increased oxidative stress causes HIV disease progression and may lead to the incidence of HIV-associated disorders. In HIV infected individuals, it is found that there is a major serum Selenium deficiency. Based on available studies, it appears as though selenium is being utilized in a form that cannot be recycled during the course of HIV disease leading to lower serum selenium levels in HIV positive individuals. Antioxidant proteins such as catalase and

glutathione peroxidase, which require selenium to function, were shown to decrease viral activation. While the role of selenium has potential to reduce viral activation, genetic studies have shown an interplay between selenium and genes important for the propagation of HIV (89). Furthermore, research needs to be done to determine the exact association between HIV disease and oxidative stress as extenuating factors to SCAR disease progression as there are multiple outstanding clinical concerns that need to be addressed.

» Mass spec/proteomics workflow to be applied

There is a lack of protein-specific serum biomarkers for drug induced SCAR in HIV patients and identification of a protein profile during drug rechallenge could be a breakthrough in this field. Therefore, proteomic workflows will be applied to the specific patient sample set to identify and highlight specific protein and pathway functionings and dysregulations. More specifically, bottom-up relative quantification methodologies will be utilized. Bottom-up proteomics achieves protein identification by analysis of peptide fragments, generated by proteolytic digestion of intact proteins. The proteins can first be separated by GE or chromatography, in which case the sample will contain only one or a few proteins. The identity of the original protein is determined by comparison of the peptide mass spectra, with theoretical peptide masses calculated from a proteomic or genomic database. Triple time of flight mass spectrometry was selected for this immunoproteomic profiling due to the improved speed and sensitivity, in addition to no important information being lost. The increased mass resolving power and mass accuracy of time-of-flight mass analyzers help identify compounds and characterize complex mixtures. Blood is a readily accessible biofluid containing a plethora of important proteins, nucleic acids, and metabolites that can be used as clinical diagnostic tools in diseases. As the plasma from patients' blood samples will be used for proteomic workflows, there are certain considerations and challenges to factor in. For example, larger proteins may shadow smaller, more critical proteins thereby causing loss of important data analysis.

As there are no specific biomarkers or proteins of interest to identify, a data-independent (DIA) mass spectrometry methodology will be used rather than a data-dependent analysis (DDA). In a DIA analysis, all peptides within a defined mass-to-charge (m/z) window are subjected to fragmentation; the analysis is repeated as the mass spectrometer marches up the full m/z range. This results in accurate peptide quantification without being limited to profiling predefined peptides of interest.

- **Significance of this research project and research gap outlined**

Our research group and international collaborators have made strides in identifying genetic risk factors involving the HLA gene however, little to no research has been done on the protein profile of the HIV positive drug rechallenge patients. Furthermore, there is a lack of protein-specific serum biomarkers for drug induced SCAR in HIV patients and identification of a protein profile during drug rechallenge could be a breakthrough in this field. It must be noted that the immunophenotype of DRESS and SJS/TEN is poorly defined especially in the context of HIV infection. Furthermore, determining the relationship between plasma-based protein, cytokine and drug metabolite levels and the clinical characteristics of the HIV positive and negative drug rechallenge cases may lead to the development of an effective pre-screening strategy to avoid future IM-ADRs in addition to revealing specific biomarkers or pathways which will guide treatment related decisions. We hypothesize that the involvement of the adaptive immune system may drive T-cell pathogenesis, leading to severe IM-ADRs in HIV positive patients.

Several research gaps with important translational significance remain in the context of HIV-associated DRESS to anti-TB drugs. Particularly, the diverse phenotypes and occurrence of patients reacting to one or more than one anti-TB drugs which offer unique settings to tease apart some of the immune mechanisms at play. The fundamental question being to try to find biomarkers that might help difference HLA-restricted T-cell reactive to specific pharmacological agents from non-specific transient immune reactions without lasting immunological memory. This would have significant clinical implications for ensuring this vulnerable patient population receives life-saving therapy without the unnecessary exclusion of critical drugs. The current lack of mechanism work justifies the use of a hypothesis-generate proteomic approach. Sampling the plasma proteome will allow for the identification of key immune pathways that are differential activated in the context of HIV/TB-associated DRESS and during *in vivo* drug exposure.

– Aims and Objectives

Hypothesis:

The differing clinical phenotypes of reactions during anti-TB sequential drug rechallenge reactions, in HIV/TB co-infected patients with FLTD-associated DRESS will have unique immune proteomic signatures. A distinct set of protein biomarkers may help differentiate non-specific flare-up reactions that could be treated through from reactions with immunological memory.

Aim 1:

To characterize the plasma immune proteome following *in vivo* drug provocation in a well-characterised cohort of HIV TB co-infected patients with treatment-limiting DRESS to first-line anti-TB treatment

Objective 1: Describe the plasma immune proteome changes over time amongst anti-TB drug DRESS cases in TB HIV co-infection (acute DRESS vs drug rechallenge reactions)

Objective 2: Compare plasma immune proteome between acute samples and positive drug provocation samples, stratified by offending drug or drug metabolite, clinical features of the rechallenge reaction, HLA, and in vitro cellular responses to drug.

Objective 3: Compare plasma immune proteomes of DRESS patients with positive drug provocation samples in patients with clinical reactions to a single or multiple of the FLTDs

– Methods

- **Ethical considerations**

The parent study and biorepository are conducted in accordance with the Declaration of Helsinki. This sub-study will work with de-identified samples from the biorepository and thus patient confidentiality is not an issue. All experiments will be conducted according to GLP and with HREC approval. Furthermore, annual approvals will be maintained. Ethics approval for this laboratory study was obtained from the University of Cape Town (UCT), Faculty of Health Sciences Human Research Ethics Committee (HREC 377/2019). All participants (cases and controls) considered for this study were registered with the parent IMARI Africa registry and biorepository (HREC R031/2018).

- **Study design and selection of participants**

This study is a sub-study of the IMARI Africa registry and biorepository (HREC R031/2018). The study focuses on the two major phenotypes of SCAR – SJS/TEN and DRESS. The focus will be on HIV infected patients with reactions to first-line anti-TB drugs as these form the majority of cases in the IMARI registry; other cases and samples may be used as controls and for comparison.

Extensive clinical details were required when identifying which patients to include in the patient cohort for this particular study and analysis. In order to effectively characterize all the clinical details and sampling times of each patient, patient timelines are drawn up. This depicts the critical events that took place for the patients over the course of their hospital admission and drug rechallenge. The criteria for inclusion of patients for this specific study cohort included the following;

- HIV and TB co-infection
- Definite/possible/probable DRESS phenotype
- Single/multiple reactor to first line TB medication

- Sequential drug challenge patient and;
- Similar sampling times (baseline/acute, positive reaction and recovery samples)

Table 2.1: Inclusion and exclusion criteria for SCAR patients recruited for the IMARI registry

Inclusion criteria	Exclusion criteria
Age ≥ 12 years old	Unable to provide written informed consent/assent
Written informed consent/assent provided	Drug treatment not stopped/interrupted with resolution of clinical/laboratory abnormalities
Clinical and laboratory phenotype consistent with SCAR	No histology available
Drug(s) treatment stopped/interrupted due to clinical/laboratory abnormalities	Naranjo or Alden (SJS/TEN) probability scores not suggestive of a specific drug-ADR
Routine histological evidence of immune-mediated pathology (skin)	Patient on oral/intravenous corticosteroids or other immunosuppressive medications within four weeks of presentation
Naranjo or Alden (SJS/TEN) probability scores suggests “probable”, “very probable” or “definite” for a specific drug-ADR	HIV status unknown with patient refusing testing
Causative drug has been identified during drug provocation tests	Unable to provide written informed consent/assent
HIV status confirmed	Drug treatment not stopped/interrupted with resolution of clinical/laboratory abnormalities

The following cases will be required to meet the specific aims of the project:

Proteomics work:

1. HIV/TB infected DRESS with pre- and post-positive drug challenge samples (single reactor)
2. HIV/TB infected DRESS with pre- and post-positive drug challenge samples (multiple drug reactor)

The following table outlines the patients used within the study in further detail in an attempt to conceptualize the reasoning behind the selection process of these participants. As noted below, all participants in the study were HIV positive, had a probable/definite DRESS phenotype and all reacted to first line TB medication. This was purposefully done to maintain consistency for downstream proteomic profile comparison. It must also be noted that within this sample set, there are patients that react to a single drug i.e., single reactors and patients that react to multiple drugs i.e., multiple reactors. This is to investigate the differences between these two sample groups in an attempt to characterize the changes in pathways or protein levels to answer the question of why some patients react to one drug while others react to multiple.

Table 2.2: A table outlining the patient cohort, cases and controls, and the corresponding sampling times and reactions of each patient

Patient Number	Sampling Time	Reaction
10001	13-Feb-19	Acute
	01-Mar-19	+ drug reaction RIF
	19-Mar-19	+ drug reaction delayed
	10-Apr-19	Recovery
	24-Nov-20	18 months follow-up
10085	01-Nov-19	Acute
	20-Nov-19	+ drug reaction RIF
	17-Dec-19	+ drug reaction Rifabutin
10092	29-Nov-19	Acute
	06-Dec-19	Pre-rechallenge
	05-Feb-20	Recovery

10015	04-Feb-19	Acute
	11-Feb-19	Pre-rechallenge
	19-Feb-19	+ drug reaction INH
	30-Apr-19	3 months follow-up
10051	20-Jun-19	Acute
	19-Jul-19	+ drug reaction PZA
	30-Jul-19	+ drug reaction EMB
10071	29-Aug-19	Acute
	11-Nov-19	+ drug reaction PZA
	07-Feb-20	6 months follow-up
	16-Apr-21	20 months follow-up
	10-Jun-21	22 months follow-up
	06-Aug-21	24 months follow-up
	17-Sep-21	25 months follow-up
10100	22-Jan-20	Acute
	16-Oct-20	11 months follow-up
10104	29-Jan-20	Acute
	26-Feb-20	+ drug reaction PZA
	11-Sep-20	8 months follow-up

It is critical to note that each patient included in this study, and the larger IMARI study, has been sampled multiple times throughout their disease state and drug provocation as depicted in *Table 2*. To accurately characterize the proteomic dysregulation in each patient, analysis will be done on all stages throughout the patient's disease. An acute and recovery sample has been taken for all patients which will enables the ability to tease out recovery from acute states.

- **Validation of DRESS using RegiSCAR**

In order to define a DRESS case more accurately, the careful diagnosis needs to take place. The diagnosis of DRESS syndrome requires vigilance and careful clinical observations, and a thorough laboratory examination. RegiSCAR constitutes a European registry of severe

cutaneous adverse reaction (SCAR), including Stevens-Johnson syndrome, toxic epidermal necrolysis, acute generalized exanthematous pustulosis, and DRESS. One of the aims of this registry is to delineate each of these SCARs as distinct entities. In this line, the RegiSCAR's scoring system has been designed to grade DRESS cases as "no," "possible," "probable," or "definite" case (90, 91). In order for a DRESS case to be diagnosed, specific RegiSCAR criteria have to be met including (but not limited to);

- Hospitalization
- Reaction suspected to be drug-related
- Acute rash
- Fever of above 38 degrees Celsius
- Enlarged lymph nodes at a minimum of 2 sites
- Involvement of at least 1 internal organ
- Blood count abnormalities
- Lymphocytes of above or below normal limits
- Eosinophils above laboratory limits
- Platelets below laboratory limits

- **Disease burden assessments:**

  - **LAM ELISA For TB Burden Analysis**

Plasma samples collected from the IMARI patient cohort are stored at -80 degrees Celsius. These plasma samples are thawed at room temperature. This kit is based on competitive enzyme-linked immuno-sorbent assay technology. An antibody is pre-coated onto a 96-well plate. Standards, test samples, and biotin-conjugated reagent are added to the wells and incubated. A competitive inhibition reaction takes place between the biotin-labelled LAM and the unlabelled- LAM on the pre-coated antibody. The HRP-conjugated reagent is then added, and the whole plate is incubated. Unbound conjugates are removed using wash buffer at each stage. TMB substrate is used to quantify the HRP enzymatic reaction. After TMB substrate is added, only wells that contain sufficient LAM will produce a blue coloured product, which then changes to yellow after adding the acidic stop solution. The intensity of the colour yellow is inversely proportional to the LAM amount bound on the plate. The OD is measured

spectrophotometrically at 450 nm in a microplate reader, from which the concentration of LAM can be calculated (92).

› CD4 counts

In an attempt to characterize HIV patients that have differential disease burden, CD4 count is used as reliable indicator for the patient's immunological status. CD4 cells decrease with HIV disease progression and can be indirectly associated with disease burden and thus, it is used to determine the necessity for initiation of prophylactic treatment against opportunistic infections. Blood samples are retrieved from the patient and sent to the laboratory for further testing. This testing includes the preparation of the samples and the use of the flow cytometer to accurately determine the number of CD4 cells within the sample (93).

› Viral load

Viral load is a critical measurement of the volume of the specific virus that is detectable within the patient's system. While there are multiple ways to measure viral load, the most common procedure is reverse-transcriptase polymerase chain reaction (RT-PCR). RT-PCR is used to detect the presence of mRNAs, pre-mRNAs, or other types of RNA such as noncoding RNAs. The method involves using a primer annealed to the RNA of interest. For mRNA, the primer is usually a synthetic oligo(dT)15-18, a random hexamer mixture (dN)6, or a synthetic DNA oligonucleotide that is complementary to a specific transcript (a gene-specific primer). This DNA:RNA hybrid serves as a template during reverse transcription, in which the enzyme reverse transcriptase (RT) generates a single-stranded cDNA copy of a portion of the target RNA molecule. Using random hexamer priming, it is possible to obtain representative cDNA copies of sequences from the entire length of the mRNAs and pre-mRNAs in a population. This cDNA can then be used as a template for PCR. On addition of gene-specific primers, a specific DNA fragment corresponding to a portion of the RNA of interest is generated (94). The amount of cDNA can then be quantified using cycle number.

› Serology testing

Primary EBV infection was defined if there was EBV VCA IgM positivity in the acute phase of the disease. Serum EBV VCA IgM antibody was detected using an enzyme-linked immunosorbent assay (ELISA) in vitro diagnostic kit (Thunderbolt, Gold Standard Diagnostics,

CA) for the quantitative measurement of IgM antibodies against the VCA antigens p23 and p18 of EBV. Serum EBV VCA IgM positivity was defined as serum levels above the cut-off value of 0.9 index. Serum EBV VCA IgG antibody was also measured in all subjects, using the same blood samples, and serum EBV early antigen IgM and IgG and EBV Epstein–Barr nuclear antigen IgM and IgG antibodies were checked if available.

- **Drug casualty methodologies**

Drug causalty assessment reports were carried out for all possible offending drugs. These included Naranjo (DRESS) or Alden (SJS/TEN) probability scores suggesting a “possible”, “probable” or “definite” reaction for all possible offending drugs. For patients with rechallenge reactions to a specific drug, a detail of the type of rechallenge reaction was recorded. Adjunctive data including Enzyme-linked Immunospot assay (ELISpot), and HLA data were extracted, where possible to aid confirmation of most likely causative drug and phenotype. The ELISpot assay has been used as an in vitro diagnosis of offending drugs (25). This assay involves stimulation of T-cells with varying suspected offending drugs or metabolites, allowing for quantification of the number of spot-forming units or spot-forming cells that release cytokine markers (i.e., IFN-γ or IL-5) or cytolytic molecules (i.e., granzyme B or granulysin) (25).(Other in-vitro and ex vivo diagnostic tests used include lymphocyte transformation testing (LTT) and flow cytometry. An IFN-γ ELISpot assay was run on patient peripheral blood mononuclear cells (PBMCs) using optimized parent stimulating drug concentrations for Rifampicin (25μg/ml), Isoniazid (500μg/ml), Pyrazinamide (500μg/ml), and Ethambutol (500μg/ml) and Bactrim (TMP/SMX = 50/250μg/ml or 500/2500μg/ml; and 4-NIT = 10μg/ml or 100μg/ml). A positive Elispot result was considered to be greater than or equal to 50 spot forming units (SFU)/million cells (95)

› Progression of disease sampling and analysis

Within the IMARI cohort, patients are sampled at various times throughout their disease and drug provocation. The purpose behind this being the differential expression of proteins as a result of FLTB drug admission or disease state. These proteins will be studied in extensive detail to outline highlighted pathways that are dysregulated as a result of the SCAR. Consequently, blood samples are taken as a acute (i.e., no first line TB drugs present), during drug provocation, following drug provocation and a follow-up. Having the ability to see

the timeline of a patients journey from a protein expression perspective will give immense clarity on the dysregulation taking place.

- **Sample Size**

This sub-study was focused on producing high dimensionality datasets from a very well defined and uncommon patient phenotype; thus, the study was restricted to just eight patients. Each patient case consists of approximately 3 – 5 samples across the disease course, including acute/baseline, positive drug reaction and recovery). These longitudinal samples, and especially recovery sampling following resolution of acute DRESS reactions will allow separation of DRESS and drug effects from background changes to the immunoproteome related to HIV and TB disease. Proteomic signals identified in this analysis will need to be validated in larger study datasets.

- **Experimental procedures**

  › **Library generation**

    » **Plasma depletion**

A pool of plasma was made by combining 10µl of each sample. This was then used for plasma depletion using the High-Select Top14 Abundant protein depletion kit (Thermo Scientific A36369) according to the manufacturer's instructions. Briefly, 10 µL of sample is added to the resin slurry in the column until the resin is completely homogenous in solution followed by centrifugation at 1,000 × g for 2 minutes. After depletion, the flow through fractions were pooled and concentrated using filters and spinning through at 12 000 x g. The depleted fraction was then digested by in-solution digest. Briefly, samples were reduced and alkylated by the addition of 20 mM dithiothreitol (DTT; Sigma D9779) and 30 mM iodoacetamide (IAA; Sigma I6125)) followed by incubation at 60 ˚C for 10 minutes. Trypsin (Promega PRV5111) was then added at a ratio of 1:100 total protein and LysC (Pierce 90307) at a ratio of 1:250 total protein. Samples were incubated overnight (18hours) at 37°C (96).

    » **Peptide fractionation**

The peptide from the depleted and digested plasma pool was fractionated using the Pierce high pH reversed-phase peptide fractionation kit (Thermo Scientific 84868) according to the manufacturer's instructions (97). Briefly, Peptides are bound to the hydrophobic resin under aqueous conditions and desalted by washing the column with water by low-speed centrifugation. A step gradient of increasing acetonitrile concentrations in a volatile high-pH elution solution is then applied to the columns to elute bound peptides into eight different fractions collected by centrifugation. Each fraction is then dried in a vacuum centrifuge and stored until analysis by mass spectrometry. During LC-MS analysis, peptides in each high-pH fraction are further separated using a low-pH gradient, thus reducing the overall sample complexity and improving the ability to identify low-abundant peptides.

› Preparation of plasma samples

» Dilution of plasma samples for digest

In preparation for digest, a 10 µl plasma sample was diluted 5X with 2% SDS (SDS, Sigma 71736) 50mM TEAB (TEAB, Sigma T7408). Thereafter, 2 µl of each sample was processed by on-bead HILIC digest as described below.

» On-bead HILIC digest

In preparation for the HILIC magnetic bead workflow, the HILIC beads (ReSyn Biosciences, HLC010) were aliquoted into a new tube and the shipping solution removed. Beads were then washed with 250µl wash buffer (15% ACN, 100 mM Ammonium acetate (Sigma 14267) pH 4.5) for one minute. This was repeated once for a total of two washes. The beads were then resuspended in loading buffer (30% ACN, 200 mM Ammonium acetate pH 4.5) to a concentration of 2.5 mg/ml. A total of 20µg of protein from each sample was transferred to a protein LoBind plate (Merck, 0030504.100). Protein was reduced and alkylated by addition of 20 mM DTT and 30 mM IAA followed by incubation at 95 ˚C for 10 minutes. HILIC magnetic beads were added at an equal volume to that of the sample and a ratio of 5:1 total protein. The plate was then incubated at room temperature on the shaker at 900 RPM for 30 minutes for binding of protein to beads. After binding, the beads were washed four times with 500µl of 95% ACN for one minute. For digestion Trypsin, made up in 50 mM TEAB was added at a ratio of 1:20 total protein and LysC was added at a ratio of 1:250 total protein. The plate was incubated at 45˚C on the shaker for two hours. After digestion, the supernatant containing peptides was removed and dried down. Samples were then resuspended in LC loading buffer: 0.1% FA, 2.5% ACN.

» Liquid Chromatography Mass Spectrometry

LCMS analysis was conducted with a Q Exactive quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) coupled with a Dionex Ultimate 3000 nano-UPLC system. Data was acquired using Xcalibur v4.1.31.9, Chromelean v6.8 (SR13), Orbitrap MS v2.9 (build 2926) and Thermo Foundations 3.1 (SP4). Peptides were dissolved in 0.1% Formic Acid (FA, Sigma 56302), 2% Acetonitrile (ACN, Burdick & Jackson BJLC015CS) and loaded on a C18 trap column (PepMap100, 9027905000, 300 µm × 5 mm × 5 µm). Approximately 400ng of peptide was injected for each sample. Samples were trapped onto the column and washed for 3 minutes before the valve was switched and peptides eluted onto the analytical column as described hereafter. Chromatographic separation was performed with a Waters nanoEase (Zenfit) M/Z Peptide CSH C18 column (186008810, 75 µm × 25 cm × 1.7 µm) as described below. The solvent system employed was solvent A: LC water (Burdick and Jackson BJLC365), 0.1% FA and solvent B: ACN, 0.1% FA. All data acquisition was obtained using Proxeon stainless steel emitters (Thermo Fisher TFES523) (98).

The multi-step gradient (summarized in the table below) for peptide separation was generated at 300 nL/min as follows: time change 5 min, gradient change: 2 – 5% Solvent B, time change 40 min, gradient change 5 – 18% Solvent B, time change 10 min, gradient change 18 – 30% Solvent B, time change 2 min, gradient change 30 – 80% Solvent B. The gradient was then held at 80% Solvent B for 10 minutes before returning it to 2% Solvent B for 15 minutes.

Table 2.3: LC gradient for LFQ LC-MS/MS and the % used for Solvent A and B

Table 1: LC gradient for LFQ LC-MS/MS Time (min)	Flow rate (µl/min)	Solvent A (%)	Solvent B (%)
0.00	0.300	98	2
3.00	0.300	98	2
8.00	0.300	95	5
48.00	0.300	82	18
58.00	0.300	70	30
60.00	0.300	20	80

The mass spectrometer was operated in positive ion mode with a capillary temperature of 320°C. The applied electrospray voltage was 1.95 kV. Details of data acquisition are shown in the **Table 2.4** below.

Table 2.4: Mass Spectrometry data acquisition parameters full scan

Table 2: Mass spectrometry data acquisition parameters Full Scan	
Resolution	70,000 (@ *m/z* 200)
AGC target value	3e6
Scan range	350-2000 m/z
Maximal injection time (ms)	100
Data-dependent MS/MS	
Inclusion	Off
Resolution	17,500 (@ *m/z* 200)
AGC target value	1e5
Maximal injection time (ms)	50
Loop Count	10
Isolation window width (Da)	3
NCE (%)	27
Data-dependent Settings	
Underfill ratio (%)	1
Charge exclusion	Unassigned, 1, 7, 8, >8
Peptide match	preferred
Exclusion isotopes	on
Dynamic exclusion (s)	60

» **Data processing and analysis:**

Data independent acquisition (DIA) circumvents the time and abundance dependent selection for fragmentation by using predefined fragmentation windows (m/z windows) going through the whole m/z range of the previous MS1 scan. Therefore, all peptides which are present in the same m/z window at the same time are fragmented simultaneously and a MS2 spectra containing fragments from multiple peptides is acquired. Using the same m/z windows for all measurements, results in more reproducible fragmentation and potential identification across multiple measurements. However, the resulting MS2 spectra contain fragments from multiple peptides and are often more complex and do

not allow to directly link a specific (m/z) mass from the MS1 to a single MS2 fragment spectra. The main advantages include greater reproducibility and sensitivity and a greater dynamic range compared with data-dependent acquisition (DDA). However, the data analysis is complex and often requires expert knowledge when dealing with large-scale data sets.

- **Data Analysis plan**

Aim 1: Basic description of anti-TB DRESS proteome (TB HIV co-infection)

Pooled acute DRESS case samples:

Comparison of acute samples (within 1-2 weeks of onset of DRESS) of patients 10085, 10092, 10015, 10104, 10051 and 10100. Exclusion of patient 1 and 71 as these patients were sampled too long (>2 weeks) from acute symptoms onset. This analysis should highlight major immune protein's changes with acute DRESS in this cohort.

Intraindividual changes:

This analysis is related to the above analysis, but each patient will be used as their own control if they have sampling during the recovery periods. Patient samples to be included here are:

- Patient 10001 – do we see differences between the proteome of sample 13 Feb 2019 compared to sample 10 Apr 2019 and 24 Nov 2020 (the last sample should be the closest back to the patients non-DRESS acute)
- Patient 10092 – compare acute sample (29 Nov 2019) with recovery sample (05 Feb 2020)
- Patient 10015 – compare acute sample (04 Feb 2019) with recovery (30 Apr 2019)
- Patient 10071– compare acute sample (29 Aug 2019) and recovery (07 Feb 2020). Furthermore, analysis of the differences between recovery (07 Feb 2020) and 16 Apr 2021. Lastly, compare (17 Sept 2021) sample and the (16 April 2021)
- Patient 10100 – compare acute sample (22 Jan 2020) with recovery (16 Oct 2020)
- Patient 10104 – compare acute sample (29 Jan 2020) with recovery (11 Sep 2020)

Aim 2 and 3 analyses can be grouped together:

Comparison of plasma immune proteome between pre- and post-positive drug provocation samples, stratified by offending drug or drug metabolite, clinical features of the rechallenge reaction, HLA, and in vitro cellular responses to drug. Compare plasma immune proteomes of DRESS patients with positive drug provocation samples in patients with clinical reactions to a single or multiple of the FLTDs. To start the analyses involve determining what are the proteins that differ between:

- Patient 10001: Compare sample from (01 Mar 2019) and (13 Feb 2019), and then (19 Mar 2019) to (13 Feb 2019) and (19 Mar 2019) to (01 Mar 2019)
- Patient 10085: Compare samples from (20 Nov 2019) to (01 Nov 2019) and (17 Dec 2019) to (01 Nov 2019) and the (17 Dec 2019) and (20 Nov 2019)
- Patient 10015: Compare samples (19 Feb 2019) to (04 Feb 2019)
- Patient 10051: Compare samples (19 Jul 2019) with (20 Jun 2019), and (30 Jul 2019) versus (20 Jun 2019), and then compare the two positive reaction samples (30 Jul 2019) versus (19 Jul 2019)
- Patient 10071: Compare samples (11 Nov 2019) to (29 Aug 2019), see the differences if any between the (06 Aug 2021) compared to the (10 Jun 2021) samples. We can compare sample from (06 Aug 2021) to (11 Nov 2019) and this will be particularly interesting as in the first instance the patient was thought to be clinically having a reaction to PZA and then in the second they had been happily tolerated PZA therapy for 2 months
- Patient 10104: Compare (26 Feb 2020) to (29 Jan 2020) samples

› Proteomic Analysis

A study specific library was generated from a subset of fractionated samples from DRESS plasma samples as well as the actual patient plasma sample runs against a background fasta database for the human isoform reference proteome downloaded from Uniprot on 04/01/2021. This was combined with a previous study of a similar design (fractionated pool sample (n=20) + patient samples (n = 154)). Both sets of data were generated on the Evosep/6600 LC-MS system. The spectral library was generated, and subsequent data analysis performed, using

Spectronaut v15.5.211111.50606 (Rubin) (100). The settings were the standard settings and took into account the addition of LysC as an extra enzyme for digestion as well as the alkylation modification for cysteine by IAA (Carbamidomethylation of C).

› Pathway and Expression Analysis

The aim of data analysis is to translate large amounts of proteomic data that cover numerous samples, conditions and time points into structured, domain-specific knowledge that can guide clinical decisions. KEGG is an encyclopedia of genes and genomes. Assigning functional meanings to genes and genomes both at the molecular and higher levels is the primary objective of the KEGG database project. Molecular-level functions are stored in the KO (KEGG Orthology) database, where each KO is defined as a functional ortholog of genes and proteins. Higher-level functions are represented by networks of molecular interactions, reactions, and relations in the forms of KEGG pathway maps, BRITE hierarchies and KEGG modules (101). Following the proteomic analysis using the KEGG database will be used for identification of pathways and molecular interactions to further define the sample comparisons outlined. This will provide detailed insight into the molecular mechanisms and highlighted pathways during DRESS disease progress as a result of drug exposure. Furthermore, the KEGG database will allow key differences between patient samples to be highlighted. Perseus will also be used for pathway and functional analysis. Steps involved in Perseus analysis include loading the data, filtering, exploratory analysis, normalization, loading annotations, differential expression analysis, clustering and profile plots and functional analysis.

A good practice in data analysis is to start with exploratory statistics in order to check for biases in the data, undesirable outliers, and experiments with poor quality data and to make sure that all requirements for performing the subsequent statistical tests are met. Once the data are filtered and normalized appropriately, statistical and bioinformatic analyses are performed in order to identify proteins that are likely to be functionally-important. When the list of such proteins is small enough and direct links to the question of interest can be inferred using prior knowledge. However, one of the advantages of mass spectrometry-based proteomics is the ability to unravel new discoveries in an unbiased way, for instance, through functional analysis. This analysis is often based on enrichment tests, which can highlight guiding biological processes and mechanisms.

- **Statistical Analysis**

Extensive quality and control was done on the data in order to exclude any inaccurate data produced. Statistical analysis was done using Excel based data that was exported from Spectronaut and that subsequently underwent statistical analysis using the R programming language (102). Statistical analysis was done on all datasets. Expression data, metadata and feature data was compiled into a MSnSet (MSNSet_Chloe_genes.rds), using the ShinyApp MSNSet Generator and then analysed using the ShinyApp Expression Data Analyser. The Expression Data Analyser app visualises data via a variety of clustering techniques, it then performs statistical analysis and enrichment analysis on the results. Both ShinyApps are developed and maintained by Dr Shaun Garnett at the University of Cape Town on the MetaOmics server. ShinyApps were created using the shiny package in R. MSnSet data were generated using the R package MSnbase. Plots were generated using the ggplot2 from the tidyverse packages in R. Heatmaps were created using the ComplexHeatmap package in R. PLS analysis was performed using the MixOmics package in R (**full reference found at end of reference list*). Volcano plots were generated using the Enhanced Volcano package in R. String network plots and enrichment analysis was performed using the STRINGdb package in R. Gene Symbols were extracted from the protein database used to perform the search. Gene Symbols will be used in the analysis where possible. Where genes were identified by multiple protein isoforms the gene symbol and protein isoform will be used which will be gathered from The UNIPROT database. For example, (SERPINA1) Alpha-1-antitrypsin (103).

To analyse the difference in protein abundance, ANOVA statistical tests were used. Analysis of variance (ANOVA) is one of the most frequently used statistical methods in medical research. The need for ANOVA arises from the error of alpha level inflation, which increases Type 1 error probability (false positive) and is caused by multiple comparisons. ANOVA uses the statistic F, which is the ratio of between and within group variances. The main interest of analysis is focused on the differences of group means; however, ANOVA focuses on the difference of variances. The illustrated figures would serve as a suitable guide to understand how ANOVA determines the mean difference problems by using between and within group variance differences.

— Results

• Patient descriptions and clinical details

Eight patients met the inclusion criteria required and their specific clinical details are described below with a HIV and TB history, timeline of drug treatments, DRESS event and sampling (**Figures 3.1 – 3.8**)

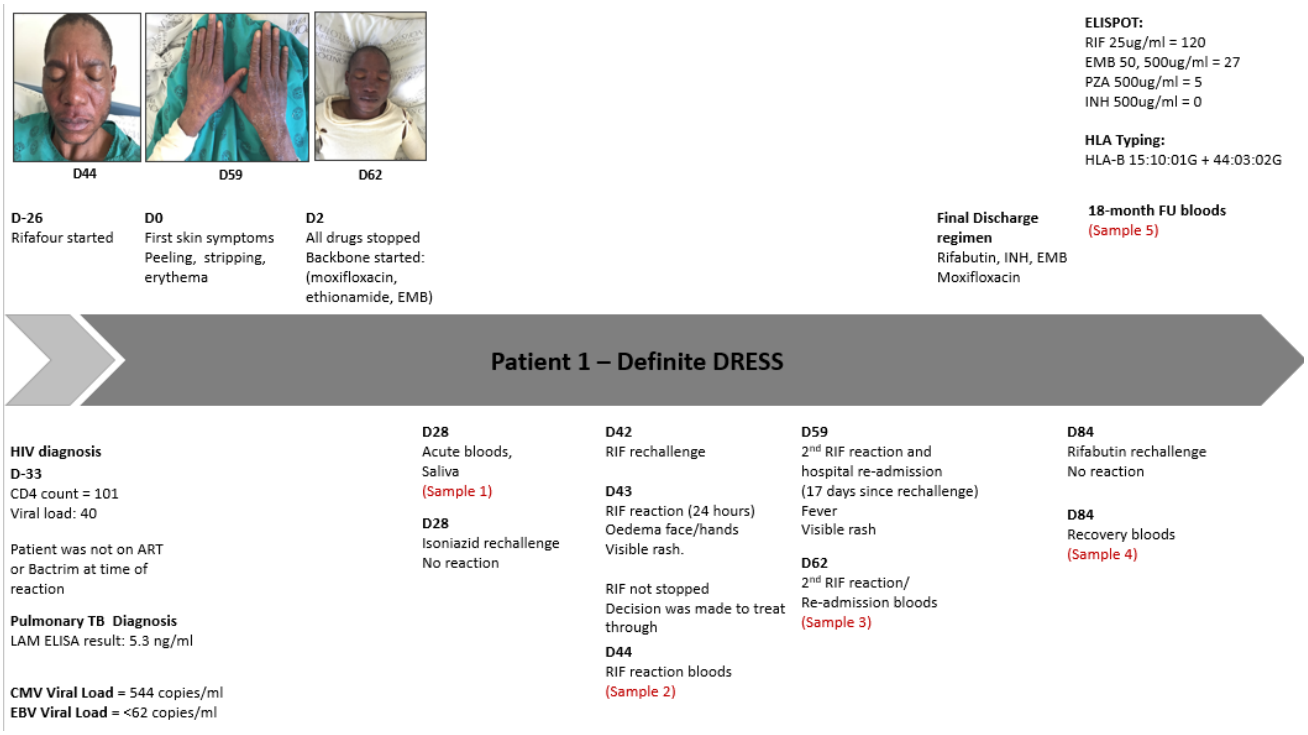


***Figure 3.1: Patient 10001 timeline*.** 37-year-old male on antituberculosis therapy. This patient is HIV and TB positive with definite DRESS to a single FLTD, Rifampicin. HIV positive since 2018, CD4 count is 101 cells/mm^3, not on ART or Bactrim at time of reaction. Pulmonary TB diagnosis in 2018, LAM ELISA result of 5.3 ng/ml. 56% Body Surface Area. Eosinophils counts (0.00 – 0.8x10^9/L) = -. Liver functioning; ALT (7 – 35U/L). First or acute blood sample for this patient included in the proteomic was 28 days after the first onset of skin rash. On Rifampicin drug rechallenge, visible rash, oedema of hands and face occurred after 24 hours. However, the clinical decision was made to treat through this early reaction and Rifampicin was only discontinued on day 17 of rechallenge with the presence of worsening rash and fever; the positive drug rechallenge sample was taken two days into the positive drug rechallenge. ELISPOT results: RIF = 120 SFU/million cells, EMB 50, 500ug/ml = 27, PZA 500ug/ml = 5, INH 500ug/ml = 0. Naranjo score drug: Discharge drug regimen: Rifabutin, INH, EMB, Moxifloxacin

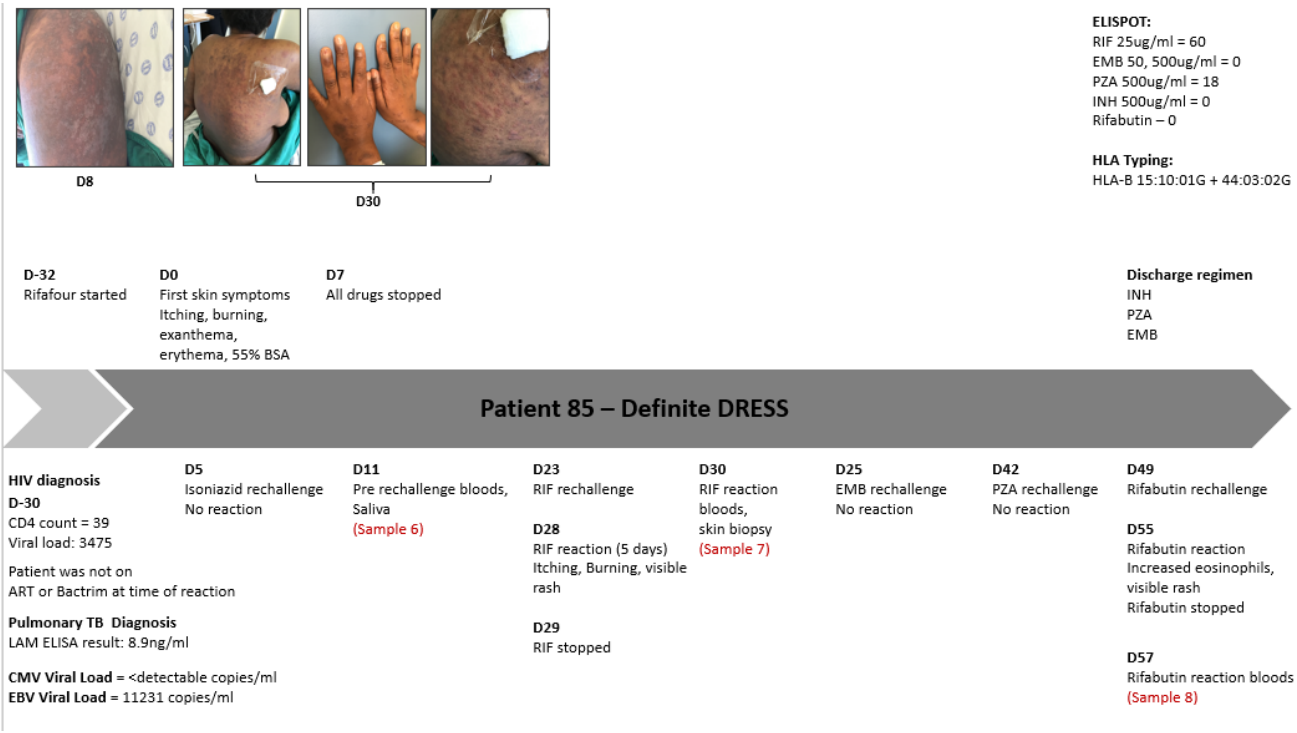


***Figure 3.2: Patient 10085 timeline*.** 43-year-old female on antituberculosis therapy. This patient is HIV and TB positive with definite DRESS to a single FLTD, Rifampicin. HIV positive since 2016, CD4 count is 39, not on ART or Bactrim at time of reaction. Pulmonary TB diagnosis in 2019, LAM ELISA result of 8.9 ng/ml. 55% Body Surface Area. Eosinophils (0.00 – 0.8x10^9/L) = 1.49, ALT (7 – 35U/L) = 68 (acute blood sample), 5 days from drug introduction to positive reaction (visible rash, itching/burning). ELISPOT results: RIF 25ug/ml = 60, EMB 50, 500ug/ml = 0, PZA 500ug/ml = 18, INH 500ug/ml = 0, Rifabutin – 0. Naranjo score: Rifampicin = 6. Positive reaction to Rifabutin. Discharge regimen: INH, EMB, PZA

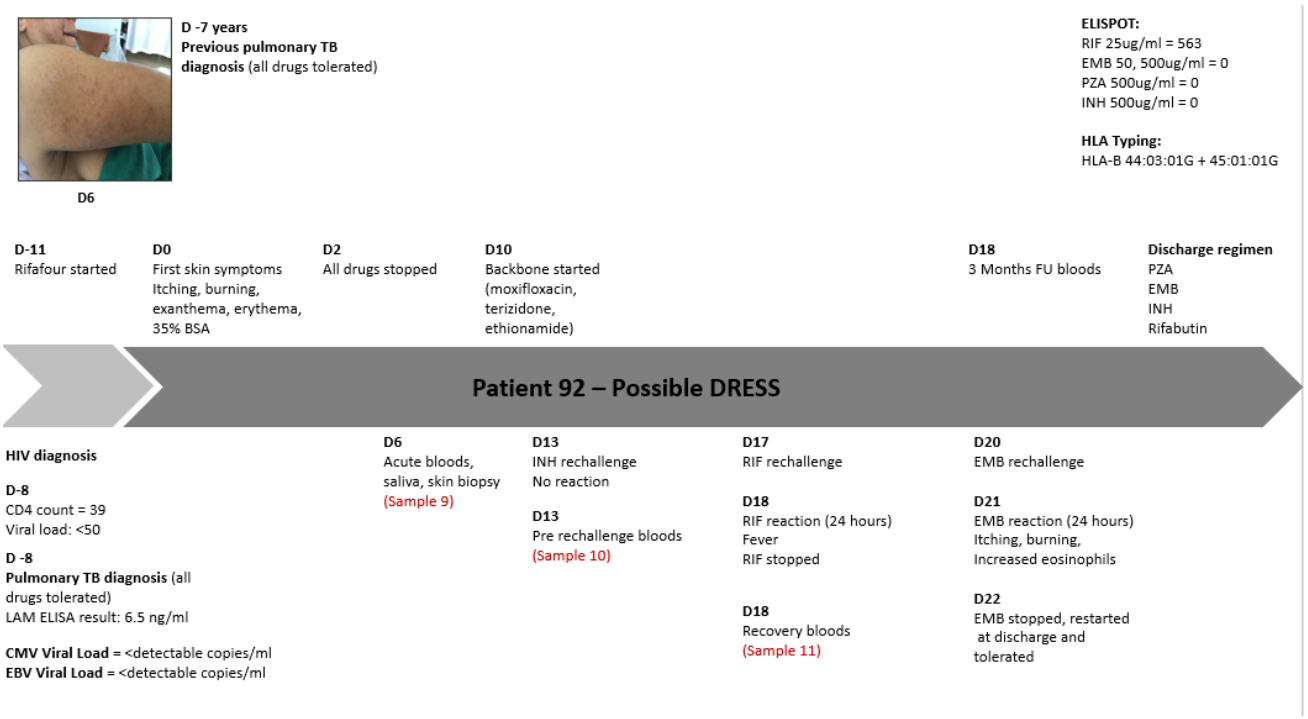


***Figure 3.3: Patient 10092 timeline*.** 39-year-old female on antituberculosis therapy. This patient is HIV and TB positive with possible DRESS to a single FLTD, Rifampicin. HIV positive since 2014, CD4 count is 39, not on ART or Bactrim at time of reaction. Extrapulmonary TB diagnosis in 2019, LAM ELISA result of 6.5 ng/ml, previous pulmonary TB diagnosis (2012). 35% Body Surface Area. Eosinophils (0.00 – 0.8x10^9/L) = 0.78, ALT (7 – 35U/L) = 65 (acute blood sample). Patient developed a fever 24 hours after starting Rifampicin and this drug was stopped, patient was commenced on rifabutin which was tolerated. A drug challenge to Ethambutol also lead to a fever and skin, itching and burning after 24 hours, but the clinical decision was made to restart this drug some days later and then the drug was tolerated for the completion of intensive phase TB treatment. ELISPOT results: RIF 25ug/ml = 60, EMB 50, 500ug/ml = 0, PZA 500ug/ml = 18, INH 500ug/ml = 0, Rifabutin – 0. Naranjo score: PZA = 6, EMB = 6. Discharge regimen: INH, EMB, PZA and Rifabutin

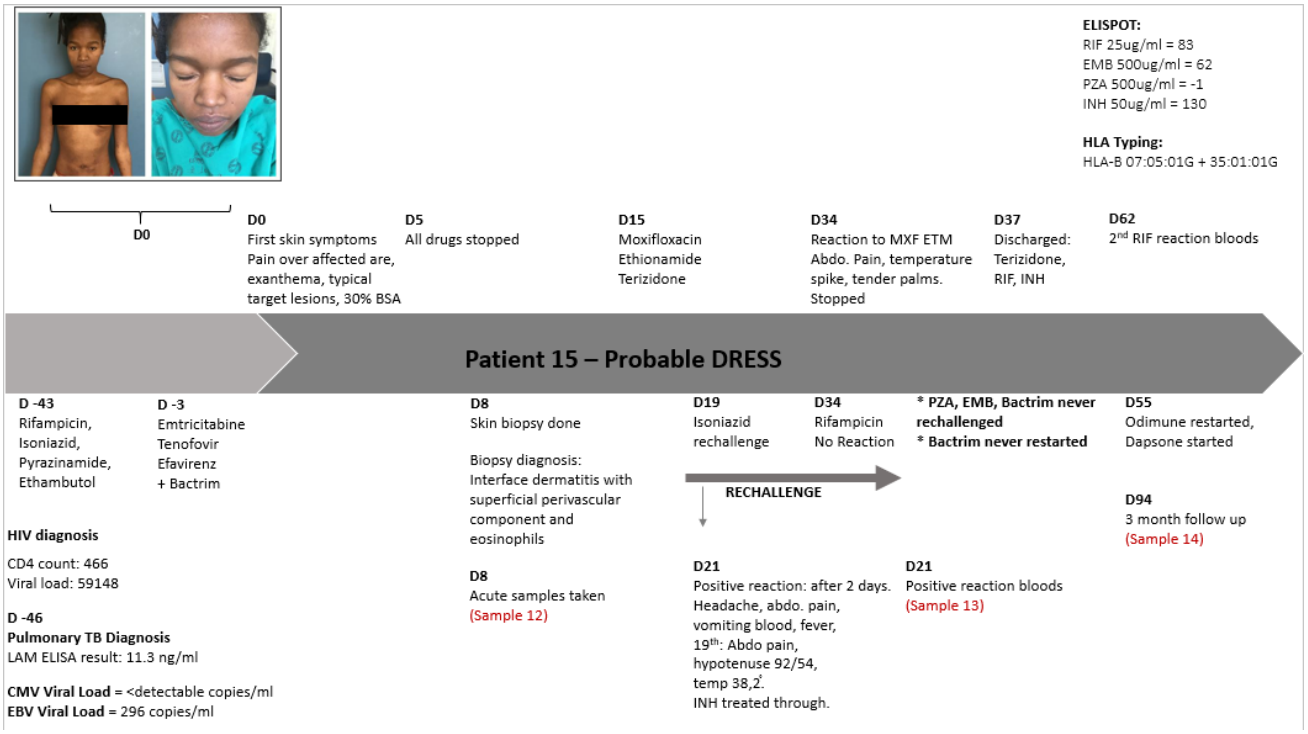


Figure 3.4: Patient 10015 timeline. 28-year-old female on antituberculosis therapy. This patient is HIV and TB positive with probable DRESS to multiple FLTDs including INH, Moxifloxacin and Ethionamide. HIV positive since 2014, CD4 count is 39, started on ART drugs three days prior to presenting with rash. Pulmonary TB diagnosis in 2018, LAM ELISA result of 11.3 ng/ml. 30% Body Surface Area. Eosinophils (0.00 – 0.8x10^9/L) = 0.29, ALT (7 – 35U/L) = 163 (acute blood sample). Drug rechallenges resulted in two reactions, the first involved fever, abdominal pain, headache with onset 24 hours following INH introduction; this reaction was treated through and the patient was discharged on INH. Patient developed a similar type of reaction to moxifloxacin or ethionamide with fever, abdominal pain, and palmar tenderness; bridging therapy was stopped and not rechallenged due to available preferred alternatives. Liver function severity meant it was decided to not rechallenge PZA or ETH from the first-line anti-TB drugs. ELISPOT results: RIF 25ug/ml = 83, EMB 500ug/ml = 62, PZA 500ug/ml = -1, INH 50ug/ml = 130. This patient was not rechallenged on to several drugs that she was exposed to during the latency period, thus Naranjo score were possible for several drugs including Bactrim = 4, INH = 4, MXF = 4, ETH = 4, and RIF = 3. INH positive reaction but decision made to treat through. PZA, EMB never rechallenged. Bactrim never restarted. Discharge regimen: Terizidone, RIF, INH

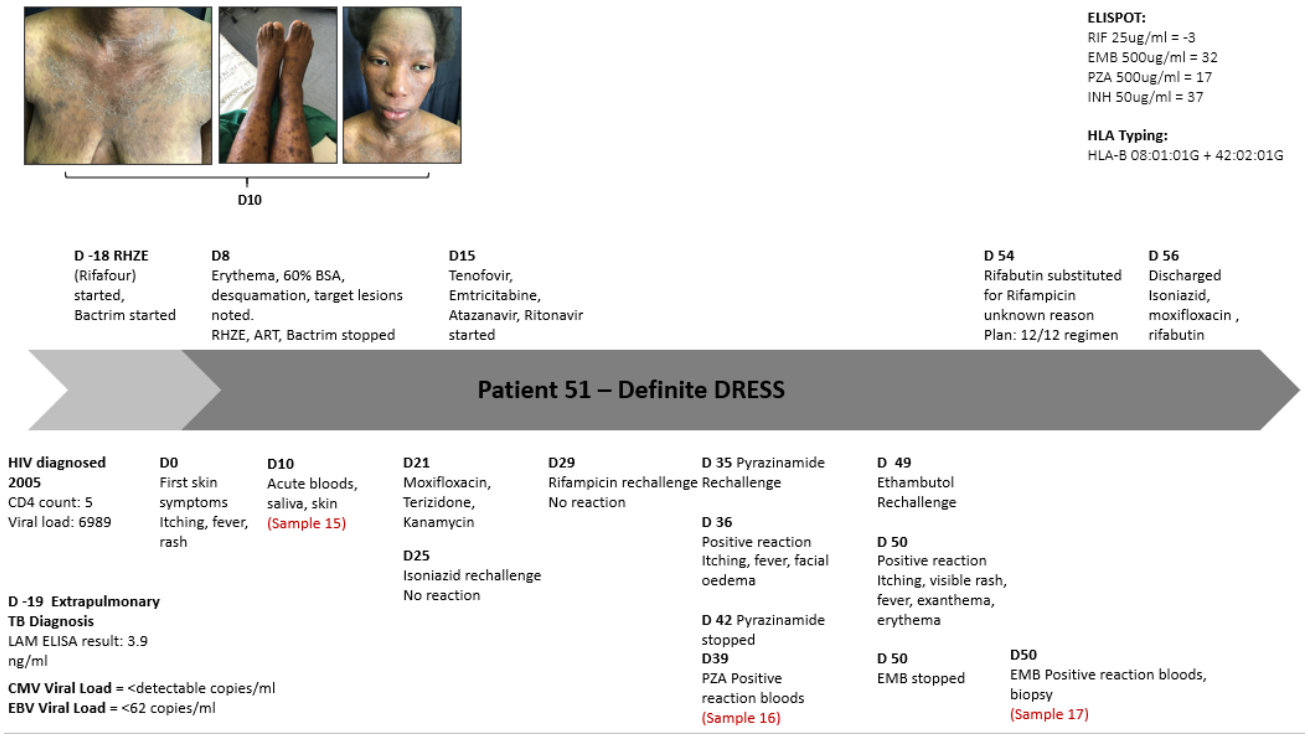


***Figure 3.5: Patient 10051 timeline*.** 28-year-old female on antituberculosis therapy. This patient is HIV and TB positive with definite DRESS to multiple FLTD, Pyrazinamide and Ethambutol. HIV positive since 2005, CD4 count = 5, Viral Load = 6989 not on ART at time of reaction but was on Bactrim. Extrapulmonary TB diagnosis in 2019, LAM ELISA result of 3.9 ng/ml. previous pulmonary TB diagnosis (2016) RHZE (Rifafour) used with no IM-ADR. 60% Body Surface Area. Eosinophils (0.00 – 0.8x10^9/L) = 2,74, ALT (7 – 35U/L) = 65 (acute blood sample), 24 hours from drug introduction to positive reaction (Itching, fever, facial oedema) for Pyrazinamide which continued; sample taken three days from start of onset of drug rechallenge symptoms. Ethambutol rechallenge resulted in Itching, visible rash, fever, exanthema, erythema 24 hours after starting therapy, sampling occurred soon after the onset of the reaction. ELISPOT results: RIF 25ug/ml = -3, EMB 500ug/ml = 32, PZA 500ug/ml = 17, INH 50ug/ml = 37. Naranjo score: PZA = 6, EMB = 6. Rifabutin substituted for Rifampicin unknown reason. Discharge regimen: Isoniazid, Moxifloxacin, Rifabutin

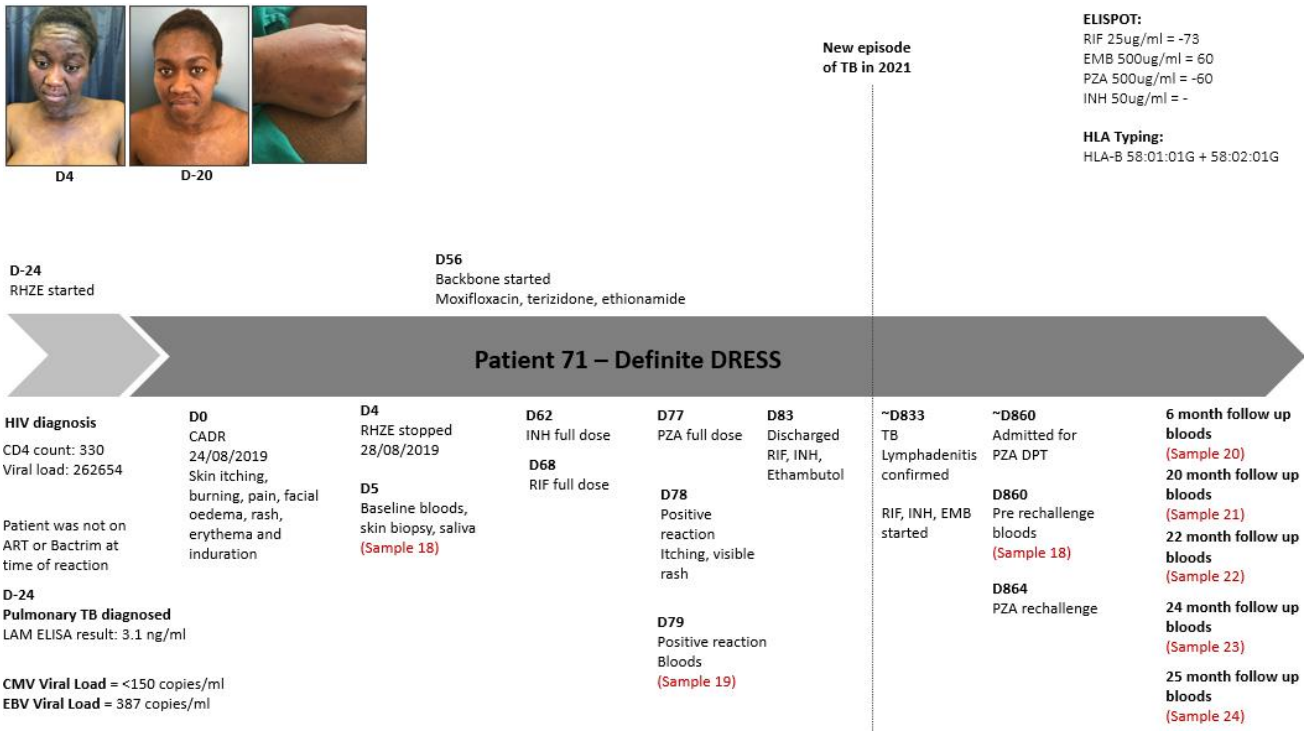
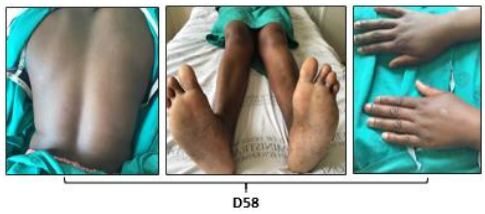


Figure 3.6: Patient 10071 timeline. 28-year-old female on antituberculosis therapy. This patient is HIV and TB positive with definite DRESS to a single FLTD, Pyrazinamide. HIV positive since 2017, CD4 count = 330, not on ART at time of reaction but was on Bactrim. Pulmonary TB diagnosis in 2019, LAM ELISA result of 3.1ng/ml. 65% Body Surface Area. Eosinophils (0.00 – 0.8x10^9/L) = 2,91, ALT (7 – 35U/L) = 53 (acute blood sample). First-line anti-TB drug rechallenge was successful except for a positive reaction 24 hours after PZA rechallenge; this drug was stopped and in 2020 the patient was discharged on Rif, INH and Ethambutol. At follow-up in the drug allergy clinic in 2021 she was diagnosed with a new episode of TB lymphadenitis requiring treatment. She was readmitted to the ward and rechallenge with all four first-line anti-tb drugs including PZA without any rechallenge reaction. ELISPOT results: RIF 25ug/ml = -73, EMB 500ug/ml = 60, PZA 500ug/ml = -60, INH 50ug/ml = -. Naranjo score during first episode and IM-ADR: PZA = 6.



ELISPOT:
RIF 25ug/ml = 0
EMB 500ug/ml = 0
PZA 500ug/ml = 0
INH 50ug/ml = 2

HLA Typing:
HLA-B 57:02:01G + 58:01:01G

D -21
Rifafour started:
Rifampicin, Isoniazid, Pyrazinamide, Ethambutol
+ *Bactrim*

D47
Backbone
TZD, MXF, EMB

11-month FU bloods
(Sample 26)

Patient 100 – Possible DRESS

HIV Diagnosis:
D4
CD4 count = 137
Viral load = 115989

D -39
TB Diagnosis:
Pulmonary

LAM ELISA result: 11.3 ng/ml

CMV Viral Load = <detectable copies/ml
EBV Viral Load = <detectable copies/ml

D -6
3rd line ART
Tenofovir
Emtricitabine
Darunavir
Ritonavir

D0
First skin symptoms: Peeling, erosions, erythema, lip and oral mucosa, 65% BSA

D4
All drugs stopped

D54
Isoniazid

D57
Positive reaction: 72 hrs
Visible rash, erythema, fever, joint pain, nausea
21/01/20

D58
INH stopped

D58
INH reaction bloods, skin biopsy

D58
Acute bloods
(Sample 25)

D63
Rifabutin, Pyrazinamide, Ethambutol
No reaction

D63
Discharged
MXF, PZA, EMB
Rifabutin

***Rifampicin**
Not rechallenged

D74
Follow Up bloods

***Figure 3.7: Patient 10100 timeline*.** 32-year-old female on antituberculosis therapy. This patient is HIV and TB positive with definite DRESS to a single FLTD, Isoniazid. HIV positive since 2004, CD4 count = 137, on ART and Bactrim at time of reaction. Pulmonary TB diagnosis in 2019, LAM ELISA result of 11.3 ng/ml. 65% Body Surface Area. Eosinophils (0.00 – 0.8x10^9/L) = 1,04, ALT (7 – 35U/L) = 13 (acute blood sample), 72 hours from drug introduction to positive reaction (Visible rash, erythema, fever, joint pain, nausea) for Isoniazid. ELISPOT results: RIF 25ug/ml = 0, EMB 500ug/ml = 0, PZA 500ug/ml = 0, INH 50ug/ml = 2. Naranjo score: INH = 6. Rifampicin not rechallenged. Discharge regimen: MXF, PZA, EMB, Rifabutin

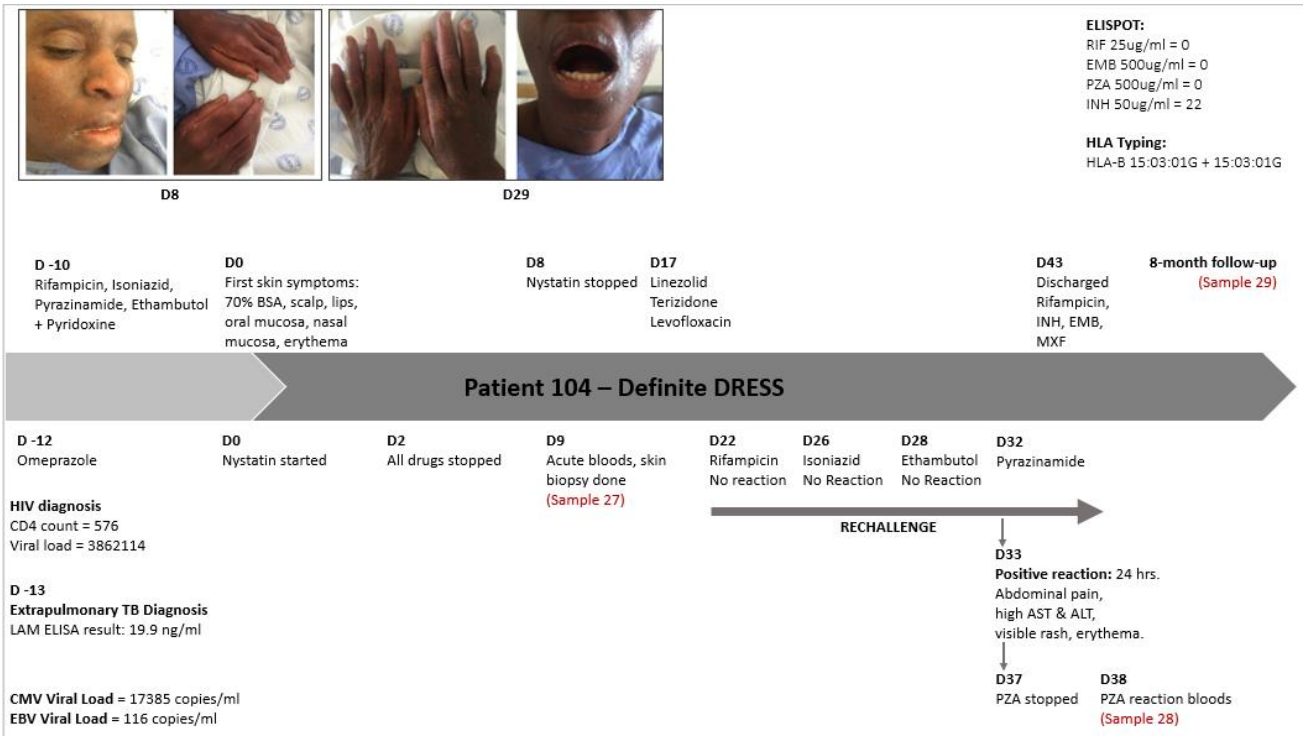


***Figure 3.8: Patient 10104 timeline*.** 48-year-old male on antituberculosis therapy. This patient is HIV and TB positive with definite DRESS to a single FLTD, Pyrazinamide. HIV positive since 2014, CD4 count is 578, log Viral Load = 6.59 not on ART at time of reaction but was on Bactrim. Extrapulmonary TB diagnosis in 2019, LAM ELISA result of 19.8 ng/ml. 70% Body Surface Area. Eosinophils (0.00 – 0.8x10^9/L) = 0.19, ALT (7 – 35U/L) = 61 (acute blood sample), 24 hours from drug introduction to positive reaction (Abdominal pain, high AST & ALT, visible rash, erythema) for Pyrazinamide. ELISPOT results: RIF 25ug/ml = 0, EMB 500ug/ml = 0, PZA 500ug/ml = 0, INH 50ug/ml = 22. Naranjo score: PZA = 4. Readmission 1 week after discharge for jaundice, fever, vomit, Pyrexial. TB medication stopped and Piptaz & Amikacin started. Discharge regimen: Rifampicin, INH, EMB, MXF

Table 3.1: A table summarizing specific clinical details of the eight patients included in the study

Patient Number	Gender	HIV status	TB status	SCAR Phenotype	Single/Multiple reaction	Positive reaction drugs	Highest Naranjo Scoring Drug	Latency	Positive reaction latency
10001	M	+ CD4: 101 VL: 40	+	Definite DRESS	Single	RIF	RIF 6	RIPE: 26 days	RIF: 24 hours
10085	F	+ CD4: 39 VL:3475	+	Definite DRESS	Single	RIF/Rifabutin	RIF: 6	RIPE: 32 days	RIF: 5 days, RIFB: 6 days
10092	F	+ CD4: 39 VL:<50	+	Possible DRESS	Single	RIF	RIF: 6	RIPE: 11 days	RIF:24 hours
10015	F	+ CD4: 466 VL:59148	+	Probable DRESS	Multiple	INH, MXF, ETH	Bactrim:4 INH = 4 MXF = 4 ETH = 4 RIF= 3	RIPE: 42 days, Bactrim: 3 days	INH: 48 hours MOX/Ethio: 2day
10051	F	+ CD4: 330 VL:6989	+	Definite DRESS	Multiple	PZA, EMB	PZA: 6 EMB: 6	RIPE: 18 days, Bactrim: 32 days	PZA: 24 hours, EMB: 24 hours
10071	F	+ CD4: 330 VL:262654	+	Definite DRESS	Single	PZA	PZA: 6	RIPE: 25 days	PZA: 48 hours
10100	F	+ CD4: 137 VL:115989	+	Possible DRESS	Single	INH	INH: 6	RIPE, Bactrim:20 days	INH: 72 hours
10104	M	+ CD4: 578 VL:3862114	+	Definite DRESS	Single	PZA	PZA: 6	RIPE: 10 days	PZA: 24 hours

In addition to the clinical details portrayed above, two other details regarding these eight patients are important to include in this analysis: liver functioning and body surface area.

Table 3.2: A table summarizing specific liver functioning and body surface area affected for the eight patients included in the study

Patient Number	ALT (u/L)	AST (u/L)	Body Surface Area (%)
10001	2333		56
10085	68	118	55
10092	65	96	35
10015	163	187	30
10051	65	80	60
10071	49	54	65
10100	13	22	65
10104	61	92	70

*Acute numbers

- **Disease burdens of HIV, TB and chronic herpes viruses during DRESS reactions and proteomic blood sampling**

› HIV Viral Load and CD4 count

While DRESS has been described to be life-threatening illness, it is particularly problematic in the context of HIV TB co-infection (21). DRESS patients co-infected with HIV/TB results in them being at an incredibly high risk for opportunistic infections. Interestingly, some patients report tolerance to anti-TB drugs prior to the development of HIV but develop SCAR on subsequent exposures to first-line anti-TB drugs (FLTB) when co-infected with HIV, TB-HIV co-infected patients can ill afford treatment interruptions, which occur as a consequence of SCAR. Identifying the culprit drug is a major challenge in the context of 4-8 drug polypharmacy (22). Thus, any treatment interruptions are life-threatening and need to be minimised. Furthermore, TB is a complex disease to treat, requiring multiple drugs for prolonged period (23). Most patients included in the study had uncontrolled HIV with detectable viral loads. There were variable CD4 counts with patients 10100 and 10104 having the highest CD4 counts (and HIV viral loads).

› TB disease burden

Mycobacterial infections and active TB have been shown to impact immune responses, thus could be important co-factors in DRESS pathogenesis to FLTD. Mycobacterial disease burdens can be measured through number of days to sputum culture positivity (few days equates to higher sputum bacillary burden), cycle threshold values with GeneXpert MTB (low Ct values correspond to higher sputum bacillary loads). If patients are culture positive in non-sputum samples e.g., blood, urine, or lymph node, they have disseminated TB (more common in advanced HIV), in this context blood culture time or positive or LAM ELISA can be used as measures of mycobacillary burden.

› LAM ELISA

LAM ELISA is a measure of lipoarabinomannan – a mycobacterial cell wall component; is released into the circulation with breakdown of mycobacteria. All patients had serum sample LAM ELISA measurements to quantify relative systemic mycobacterial burden. **Figure 3.9** shows LAM concentrations across patients. The detectable LAM in all the patient samples indicates that all patients likely had TB disease beyond the lungs, as isolated pulmonary TB is very uncommonly associated with positive serum LAM. Relating these LAM ELISA measurements to clinical phenotyping notes there is: i) no clear pattern between CD4 count and LAM ELISA, in fact patients 104 and 10015 had the highest CD4 counts and also have the highest concentration of serum LAM; ii) the three patients with the highest serum LAM (10104, 10015 and 10100) included two single drug reactors (100100 and 100104) and one multiple drug reactor (10015), and iii) two patients had a clinical diagnosis of extrapulmonary TB (10051 and 10104) and only 104 had very high serum LAM; notably several of the other patients with reported isolated pulmonary TB had detectable serum LAM likely reflecting undiagnosed dissemination of TB.

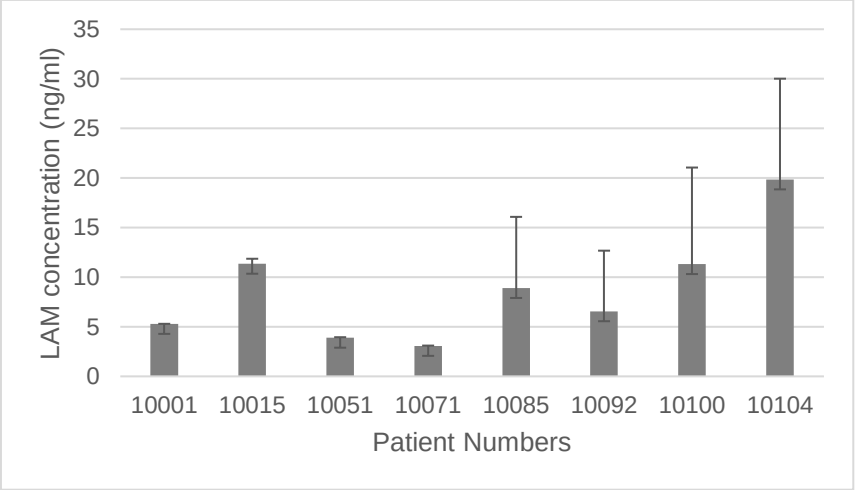


Figure 3.9: Tuberculosis burden determined by LAM ELISA analysis. *Error bars show standard deviation.*

Table 3.3: Duplicated OD readings for each patient number and the calculated corresponding LAM concentration. Calculated using a standard curve.

Patient Number	OD readings 1*	OD readings 2*	LAM Concentration (ng/ml)

10001	0,473	0,475	5,3
10085	1,047	1,119	11,3
10092	0,33	0,338	3,9
10015	0,249	0,254	3,1
10051	0,328	1,347	8,9
10071	0,168	1,036	6,6
10100	0,388	1,771	11,3
10104	2,658	1,215	19,8

**OD reading minus blank*

› Herpes viral reactivation

Viral reactivation to herpes viruses is known to occur in the context of HIV and DRESS. In order to assess the impact of certain confounding factors including Cytomegalovirus (CMV), and Epstein-Barr Virus (EBV) in the sample set, LAM ELISA testing was performed. Epstein Barr Virus more formally called Human Gammaherpesvirus-4, and Cytomegalovirus are two of the most common viruses in humans. While infection with CMV results in mild symptoms almost indistinguishable from EBV including fever, lymphadenopathy and pharyngitis, the indirect effects of these viruses on patients taking first line TB medication remains unknown. After analysing the presence of the Epstein-Barr virus in the sample set, it was noted that all patients had evidence of past infection with CMV and EBV illustrated by positive acute serology (IgG) to these viruses. Then viral reactivation has been linked to DRESS symptoms and clinical flaring. There is evidence of prior infection amongst everyone so simply having infection is insufficient to drive any of the differences between these patients. However, the way in which these viruses are being reactivated could actually be a very important driver or co-factor of intraindividual plasma protein changes.

Table 3.4 CMV and EBV viral loads for all samples included in the study

Patient Number	Sample type (days post skin symptoms)	EBV Viral Load (copies/ml)	CMV Viral Load (copies/ml)
10001	Acute (28)	544	<62
	Drug reaction RIF (44)	<detectable limit	203
	Drug reaction delayed (62)	<detectable limit	290

	Recovery (84)	288	5318
	18-month follow-up (~730)	4196	<detectable limit
10085	Acute (11)	<detectable limit	11231
	Drug reaction RIF (30)	<detectable limit	20180
	Drug reaction Rifabutin (57)	<detectable limit	1233
10092	Acute (8)	<detectable limit	<detectable limit
	Pre-rechallenge (15)	<150	<62
	Recovery (78)	<150	923
10015	Acute (8)	<detectable limit	296
	Drug reaction INH (21)	<detectable limit	1738
	3-month follow-up (90)	<detectable limit	1284
10051	Acute (10)	<detectable limit	<62
	Drug reaction PZA (39)	<150	1092
	Drug reaction EMB (50)	<detectable limit	935
10071	Acute (5)	<150	387
	Drug reaction PZA (79)	6693	<detectable limit
	6-month follow-up (~180)	959	92
	20-month follow-up (~600)	1266	<62
	22-month follow-up (~660)	1346	180
	24-month follow-up (~730)	6385	<62
	25-month follow-up (~750)	577	<detectable limit
10100	Acute (58)	<detectable limit	<detectable limit
	11-month follow-up (~330)	2161	<detectable limit
10104	Acute (9)	17385	116
	Drug reaction PZA (38)	601	266244
	8-month follow-up (~240)	<detectable limit	<detectable limit

- **Proteomic analysis**

  - **Quality Control processes**

Using the program, Spectronaut, Quality Control on the Mass Spectrometry plasma sample analysis data was performed. This proteomics data analysis program allows for deductions to be made regarding the quality of the data acquired as well as whether any data is missing or outliers present. Several visualizations are presented to depict the data in different ways in order to say, with certainty, that the data has been collected correctly, contains no handling errors and is able to be analysed accurately. These figures (figures S1 – S4) have been included in the supplementary information section at the end of the dissertation.

  - **Differential expression**

Plasma samples from 8 patients were selected to perform data-independent acquisition-based label free discovery mass spectrometry on. There were various groups in which comparisons were made including:

1. **Overview** of the samples to investigate overall clustering and highlighted proteins across all samples
2. **Analysis 1:** Acute sample analysis to assess the interindividual regulation of proteins (patients 10001 (1), 10085 (6), 10092 (9), 10015 (12), 10051 (15), 10071 (18), 10100 (27) and 10104 (29))
3. **Analysis 2:** Acute versus recovery samples to highlight changes that occur in patients from start to end of their disease progression. Acute versus early recovery as well as acute versus late recovery will also be analysed to assess whether there is less change from the acute sample when recovery samples are taken earlier versus later (patients10001 (1), 10085 (6), 10092 (9), 10015 (12), 10051 (15), 10071 (18), 10100 (27) and 10104 (29) vs patients 10001 (4), 10092 (11), 10015 (14), 10071 (20, 23, 24), 10100 (26) and 10104 (29))
4. **Analysis 3:** Diving deeper into patients 10001 and 10071 as the clinical details of these patients are of particular interest (10001 (1, 2, 3, 4) and 10071 (18, 19, 20, 21, 22, 23, 24))

5. **Analysis 4:** Acute versus positive drug reaction looking at post-positive drug provocation samples (patients 10001 (1), 10085 (6), 10092 (9), 10015 (12), 10051 (15), 10100 (27) and 10104 (29) vs 10001 (2), 10085 (7), 10015 (13), 10051 (16, 17) and 10104 (28)).

6. The positive drug reactions will be separated into two groups: reaction 1 and 2. Reaction 1 will comprise of patients who react to a single drug (patients 10001, 10085 and 10104) while reaction 2 will comprise of patients who react to multiple drugs (patient 10015 and 10051). These reactions will both be compared to acute samples to assess immunoproteomes of DRESS patients with positive drug provocation samples in patients with clinical reactions to a single or multiple of the FLTDs.

~~7.~~6. Reaction 1 will then be compared directly with reaction 2

The statistical comparisons and differential expression were analysed using a statistical program called Perseus. Here, the data was imported and manipulated to allow for comparisons of specific samples, or groups of samples, to be made as noted in the flow diagram below. This emphasizes that the data used for statistical analysis is fit for the purpose of this study.

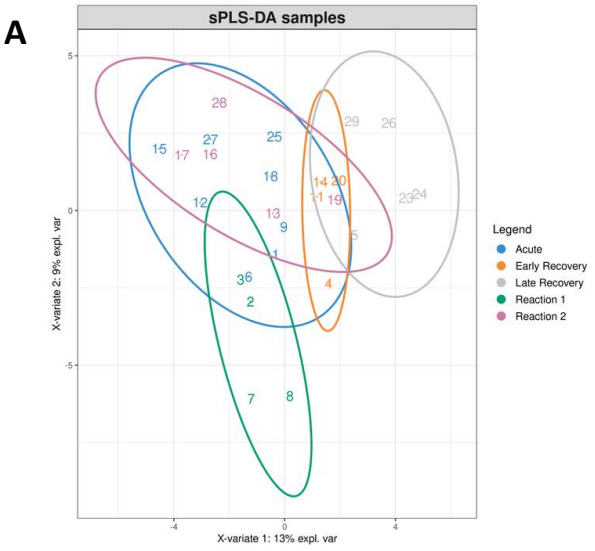
Table 3.5: Sampling numbers corresponding to the patient number and reaction type

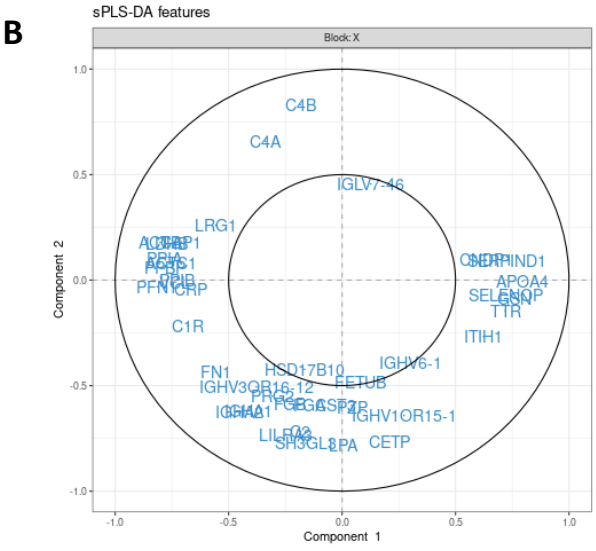
Patient Number	Sampling Date	Reaction	Sample Number
10001	13-Feb-19	Acute	1
	01-Mar-19	Drug reaction RIF (Reaction 1)	2
	19-Mar-19	Drug reaction delayed	3
	10-Apr-19	1 month follow-up (Early Recovery)	4
	24-Nov-20	18 months follow-up	5
10085	01-Nov-19	Acute	6
	20-Nov-19	Drug reaction RIF (Reaction 1)	7
	17-Dec-19	Drug reaction Rifabutin	8
10092	29-Nov-19	Acute	9
	06-Dec-19	Pre-rechallenge	10
	05-Feb-20	2-month follow-up (Early Recovery)	11

10015	04-Feb-19	Acute	12
	19-Feb-19	Drug reaction INH (Reaction 2)	13
	30-Apr-19	3-month follow-up (Early Recovery)	14
10051	20-Jun-19	Acute	15
	19-Jul-19	Drug reaction PZA (Reaction 2)	16
	30-Jul-19	Drug reaction EMB (Reaction 2)	17
10071	29-Aug-19	Acute	18
	11-Nov-19	Drug reaction PZA	19
	07-Feb-20	6-month follow-up (Late Recovery)	20
	16-Apr-21	20-month follow-up	21
	10-Jun-21	22-month follow-up	22
	06-Aug-21	24-month follow-up (Late Recovery)	23
	17-Sept-21	25-month follow-up (Late Recovery)	24
10100	22-Jan-20	Acute	25
	16-Oct-20	11-month follow-up (Late Recovery)	26
10104	29-Jan-20	Acute	27
	26-Feb-20	Drug reaction PZA	28
	11-Sep-20	8-month follow-up (Late Recovery)	29

- **Overview analysis:**

To begin the immunoproteome profiling, analysis of all samples was performed to determine clustering of patient samples/conditions based off differentially regulated proteins. The sample number that correlates with a specific patient are represented below. Moreover, pools of samples were created. These pools were required to determine if the mass spectrometry and statistical analyses was done with accuracy, thus further validating the results. Lastly, there were two further annotations made including: reaction 1 and 2, and early and late recovery. Reaction 1 and 2 refer to the positive drug reactions for the patients included in the table with reaction 1 corresponding to single drug reactions while reaction 2 corresponds to patients that reacted to multiple drugs. Early recovery corresponds to samples from patients within 3 months of the drug reaction, while late recovery are samples collected later than 3 months of the drug reaction (earliest sample 6 months from drug reaction).





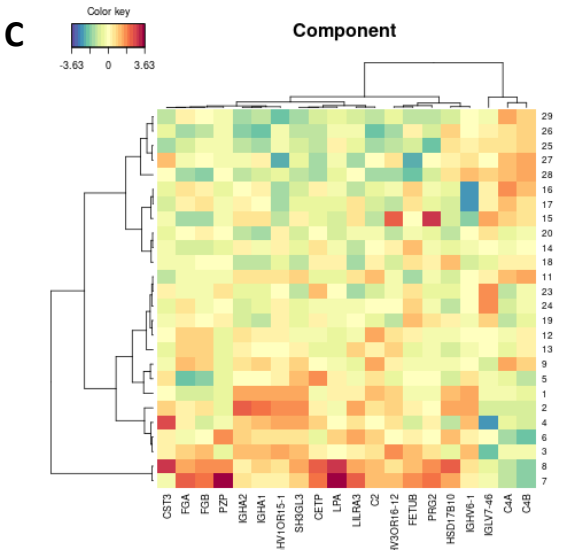


Figure 3.11: Overview analysis of all patients and sample conditions*. (A). sPLS-DA feature plots showing supervised clustering of all conditions in question namely, acute, early recovery, late recovery, reaction 1 and reaction 2, (B). a circular sPLS-DA plot showing the top 20 genes contributing to the separation of the clusters seen in (a), (C) Heatmap showing the protein expression levels of the top 20 differentiated proteins in all samples*

All the samples in the table above were included in this overview analysis in order to see if the sample groups/conditions cluster separately and which proteins drive this clustering. In **figure 3.11(A)**, a sPLS-DA (Sparse Partial Least Squares Discrimination Analysis) plot was created which clusters proteins based on their expressed/regulation relative to other proteins. It uses the proteins with the most variation to separate out the clusters. In this case, this plot is determined using the top 20 differentially regulated proteins. This statistical approach is a supervised clustering of the samples. This means that analysis of the samples is done with the condition/grouping of the samples being known. As noted in this figure, there are various condition-specific clusters forming. While there is significant overlap noted between acute, reaction 1 and reaction 2, it can also be seen that reaction 1 and 2 are separating from each other. Acute and late recovery are also separating out, albeit a small overlap. It is encouraging to see acute on the left, late recover on the right and early recovery almost connecting these which could align well with what may be happening phenotypically. The reactions also are embedded in the acute phase but in separate areas. **Figure 3.11(B)** teases out the specific proteins that contribute to the separation of the clustering seen in **Figure 3.11(A)**. The proteins in the circle are placed systematically according to how and where they influence the clustering. For example, the proteins found at the top of the

circle plot will influence the Acute and Late recovery clusters at the top of the cluster. This plot merely provides more information regarding the contributing proteins.

To provide a more careful analysis into these proteins in question, a heatmap was created (**Figure 3.11(C)**). This heatmap used all the samples in the cohort to determine the specific expression levels within each sample. These expression levels can be seen by the colour of the box corresponding to the protein/sample. The blue colour represents downregulated proteins while the dark red represents upregulation. Analysing the heatmap, separate clusters are prominent including: an overall upregulation of proteins (seen in an orange/red colour) for approximately half the patients in the bottom left corner and top right corner while downregulation (seen in a green/blue colour) noted in the bottom right corner and top left corner. All patients were included in this analysis but in no specific order, this was done to allow the samples to cluster together according to their protein expression. The samples at the top of the heatmap, samples 29, 26, 25, 27, 28, 16, 17 and 15 while samples 9, 5, 1, 2, 4, 6, 3, 8 and 7 cluster at the bottom of the heatmap. The samples at the top of the heatmap correspond to patients 10051, 10100 and 10104 while the samples clustering at the bottom of the heatmap correspond to patients 10001, 10085 and 10092. It is notable that several samples at different times in DRESS course for an individual patient appear clustered together e.g., patient 10104 samples 27, 28 and 29 cluster together on this overall heatmap but include an acute DRESS (day 9) sample, Positive drug rechallenge to PZA sample (day 33) and a late recovery DRESS sample (day 240). Intraindivudal non-DRESS factors e.g., HIV or TB disease burden factors appear to drive differences. The median (IQR) LAM concentration is higher amongst patients 104, 100, 51 versus 1, 85 and 92 [15,96 (19,85 – 3,89) versus 3,62 (8,90 – 5,28)]. No clear groupings are seen separate these two sets of patients for other clinical variables including CD4 count, HIV VL or CMV or EBV viral loads; no clear pattern is evident in ALT abnormalities. Patient 15 and 51 are the only two patients with multiple reactions to anti-TB drugs compared to 1, 85 and 92 which were all single reactors to Rifampicin.

To understand potential pathophysiological changes occurring, the pathways of the differentially regulated and expressed proteins in the clusters were investigated. Proteins (CST3) Cystatin-C, (FGA) Fibrinogen alpha chain, (FGB) Fibrinogen beta chain precursor, (PZP) Pregnancy zone protein, (IGHA2) Immunoglobulin heavy constant alpha 2, (IGHA1) Immunoglobulin heavy constant gamma 1, (IGHV1OR15-1) Immunoglobulin heavy variable 1/OR15-1, (SH3GL3) Endophilin-A3, (CETP) Cholesteryl ester transfer protein, (LPA) Apolipoprotein(a), (LILRA3) Leukocyte immunoglobulin-like receptor subfamily A member 3, (C2) Complement C2, (IGHV3OR16-12) Immunoglobulin heavy variable 3/OR16-12, (FETUB)

Fetuin-B, (PRG2) Bone marrow proteoglycan and (HSD17B10) 3-hydroxyacyl-CoA dehydrogenase type-2 are all upregulated in patients 10051, 10100 and 10104. These proteins are downregulated in patients 10001, 10085 and 10092. Proteins (IGHV6-1) Immunoglobulin heavy variable 6-1, (IGLV7-46) Immunoglobulin lambda variable 7-46, (C4A) Complement C4-A and (C4B) Complement C4-B are downregulated in patients 10051, 10100 and 10104 and subsequently upregulated in patients 10001, 10085 and 10092.

While the proteins mentioned above are upregulated in the acute/drug reaction clusters, there are a set of proteins that are downregulated in this sample group (seen in the bottom right corner of the heatmap). These proteins include (CNDP1) Beta-Ala-His dipeptidase, (GSN) Gelsolin, (APOA4) Apolipoprotein A-IV, (SELENOP) Selenoprotein P, (ITIH1) Inter-alpha-trypsin inhibitor heavy chain H1 and (TTR) Transthyretin. These proteins are subsequently upregulated in the follow-up samples. These proteins associate to common pathways to a lesser extent. One protein that is of particular interest to note here is Selenoprotein P. Selenoprotein P function in the extracellular antioxidant defence properties of selenium or might be involved in the transport of selenium.

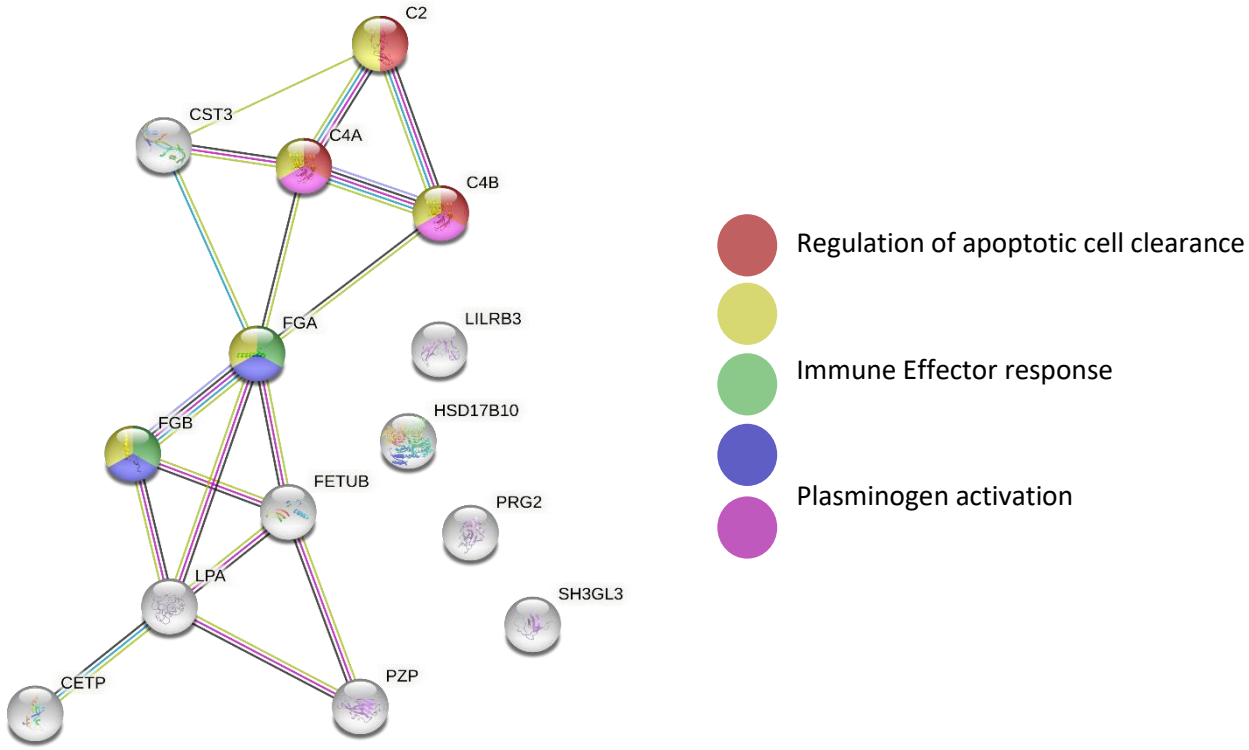


Figure 3.12: A STRING figure showing connectedness of the differentially regulated proteins and their relative interaction and pathway network. *This figure was developed using the platform, STRING (101), which outlines how these proteins are connected and the relevant pathways and cellular components that are at play. The proteins, represented as the circles known as nodes, have lines between other proteins. These lines are representative of pathways in common. The more lines between the nodes, the more significant they are for this analysis as the more pathways are highlighted. Confidence cut off of 0.5.*

Proteins in the figure include: CST3) Cystatin-C, (FGA) Fibrinogen alpha chain, (FGB) Fibrinogen beta chain, (PZP) Pregnancy zone protein, (SH3GL3) Endophilin-A3, (CETP) Cholesteryl ester transfer protein, (LPA) Apolipoprotein(a), (LILRA3) Leukocyte immunoglobulin-like receptor subfamily A member 3, (C2) Complement C2, (FETUB) Fetuin-B, (PRG2) Bone marrow proteoglycan and (HSD17B10) 3-hydroxyacyl-CoA dehydrogenase type-2, (C4A) Complement C4-A and (C4B) Complement C4-B

This string figure (**Figure 3.12**) shows connections between the 20 top differential expressed proteins in the overall unsupervised groupings. The nodes that have multiple connections are (C4A) Complement C4-A, (C2) Complement C2, (C4B) Complement C4-B, (FGA) Fibrinogen alpha chain and (FGB) Fibrinogen beta chain. It is very clear why these proteins would have pathways in common as majority of these proteins have some functional capability in the complement pathway and coagulation cascade in addition to playing a role in cell mobility, cell adhesion and cell morphology.

- **Analysis 1: Acute sample analysis to assess the interindividual regulation of proteins**

To initiate investigation into the objectives of this study, this first analysis explores the plasma immune proteome changes amongst anti-TB drug DRESS cases in TB HIV co-infection. To do this, acute samples for each patient were used to provide a more granular view on the protein changes that occur as a acute. The focus behind this analysis was to gain a deeper understanding of the major immune protein changes during acute DRESS disease progression, complexed by HIV/TB co-infection, treatment interruptions and clinical features. Apart from patients 10001 and 10100, the acute sampling time-points are very similar for all patients.

Table 3.6. Patient numbers corresponding to the sampling number and reaction type for analysis 1

Patient Number	Sample Number (days post skin symptoms)
10001	1 (28)
10085	6 (11)
10092	9 (8)
10015	12 (8)
10051	15 (10)
10071	18 (5)
10100	25 (58)
10104	27 (9)

Largescale heatmaps were created which provide insight into the expression changes that occur, as a measure of intensity. Unfortunately, there is no statistical analyses done as there are no comparisons being made (only looking at one condition). Therefore, validation of these expression levels needs to be done. As these heatmaps show all proteins in the samples, they are too large to keep in the body of this dissertation. These figures can be found in the supplementary information (**Figures S5 and S6**).

Table 3.7. Clinical details corresponding to each patient number

Patient Number	CD4+ count	HIV Viral Load	LAM ELISA (ng/ml)	Highest ALT (u/L)	Body Surface Area (%)	Days from skin symptoms onset to acute sample collection
10001	101	40	5,3	2333	56	29
10085	39	3475	11,3	68	55	11
10092	121	<50	3,9	65	35	8
10015	66	59148	3,1	163	30	8

10051	5	6989	8,9	65	60	10
10071	330	262654	6,6	49	65	5
10100	137	115989	11,3	13	65	58
10104	576	3862114	19,8	61	70	9

Figure S5 (supplementary section), differential protein expression in the acute samples, highlight proteins (SELENOP) Selenop P protein, (PRG2) Bone Marrow Proteoglycan in addition to a plethora of Immunoglobulin proteins. SELENOP was less differentiated but had a higher intensity in patients 10085, 10092, 10071 and 10100. Lastly, PRG2 had a higher intensity in patients 10051, 10071 and 10085. Selenoprotein P is responsible for the extracellular antioxidant defence properties of selenium or is involved in the transport of selenium. Lastly, PRG2 is Bone marrow proteoglycan which functions in inducing non-cytolytic histamine release from human basophils. Involved in antiparasitic defence mechanisms and immune hypersensitivity reactions. This protein is of particular interest as we are investigating immune hypersensitivity reactions in this study. The Immunoglobulins are also interesting to note as with HIV/TB, or any chronic inflammatory state, polyclonal hypergammaglobulinemia can occur in the plasma.

Figure S6 (supplementary section) investigates the expression changes that occur throughout disease progression in all samples. Like before, this heatmap shows intensity level. The specific proteins that are differentially regulated throughout the course of disease, for all patients, include (SAA2) Serum amyloid A-2 protein, (HSPB1) Heat shock protein beta-1, (CAP1) Adenylyl cyclase-associated protein 1, (HSP17B10) HSPB1-associated protein 1, (PLTP) Phospholipid transfer protein, (PPIB) Peptidyl-prolyl cis-trans isomerase B, (TRM3) tRNA (guanosine(18)-2'-O)-methyltransferase and (TPI1) Triosephosphate isomerase. All proteins have an overall higher expression in the acute samples versus the drug positive/follow-up samples across all patients.

Not only was it of interest to look at proteins that were changing throughout the disease progression for all patients, but it was also interesting to note any proteins that were vastly different between patients at similar timepoints. A reason for this being that changes between patients may indicate patient-specific reactions that are occurring that could be drug-specific, or HIV/TB specific. There are only two proteins to note here, (CRP) C-Reactive Protein and (BHMT) Betaine--homocysteine S-methyltransferase 1. BHMT is higher in intensity for acute samples for patients 10015, 10085, 10092 and 10100 whereas it is higher in follow up samples for patients 10001, 10051, 10071 and 10104. CRP has a high intensity halfway through the disease progression for patients 10001 and 10071 while it is at its highest intensity in the acute sample for patients 10015, 10051 and 10104. CRP may be correlating with the amount of TB treatment the patient has had in addition to TB disease burden as CRP is often elevated with

TB disease. BHMT is associated with acute liver injury while CRP is made in the liver in response to inflammation and functions in the innate immune response by activating the complement system.

To provide statistical validation of the results seen in the heatmaps, an ANOVA test was performed with the heatmap shown below (**Figure 3.13**). Due to the low number of samples, no multiple testing correction could be applied as there was a lack of statistical power. As seen at the top of the heatmap, there is broad clustering of the sample groups/conditions with some overlap. Proteins that are differentially regulated include CRP, CAP1, HSD17B10 which are all upregulated in the acute samples. While this does not pick up all proteins mentioned previously, it does validate that the findings for these 3 proteins and also confirms that the methodology behind the heatmaps is accurate.

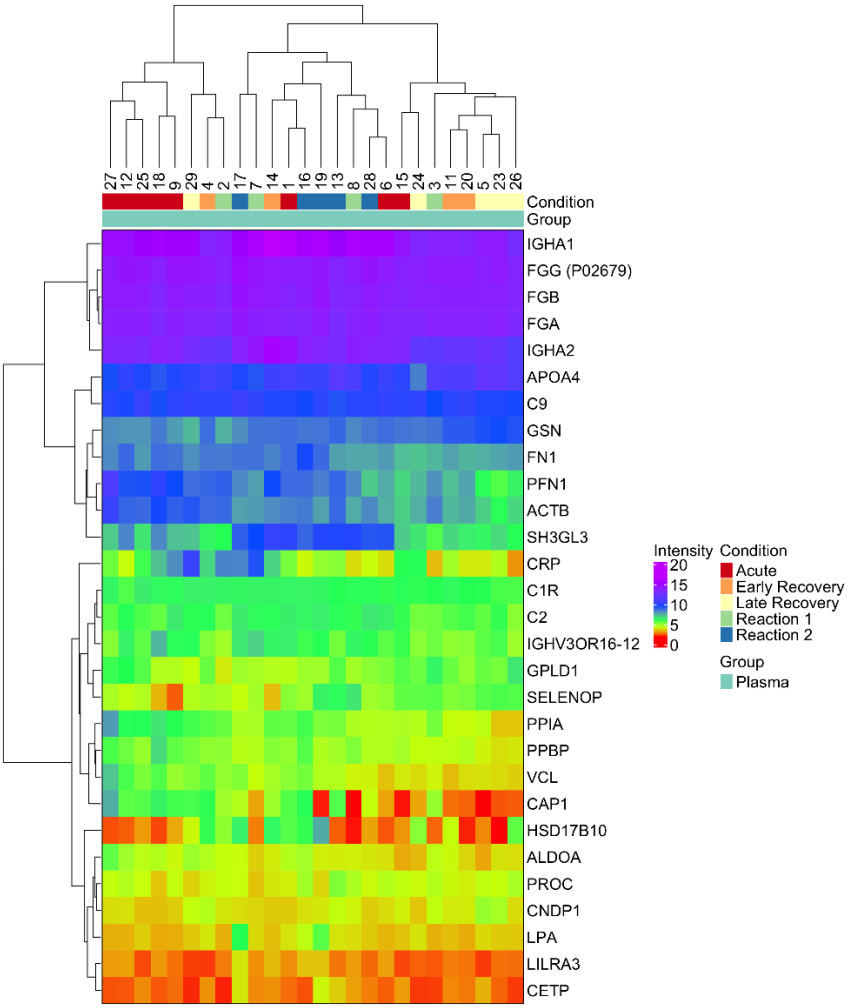


Figure 3.13: An ANOVA heatmap showing the differential expression of genes. No multiple testing correction applied.

- **Analysis 2: Acute HIV TB-associated DRESS**

Following on from the above analysis (**Figures 3.11 – 3.13**), the second part of the analysis was to investigate the changes that occur between acute DRESS and recovery samples. This was done to look at the intraindividual changes that occur within the sample group and further characterize the molecular mechanisms that occur during disease progression and help tease out the effects of DRESS disease from other HIV/TB disease and treatment changes. Patients 10001, 10092, 10015, 10100 and 10104 were analyzed within this category. The clinical background of these patients is variable. Patients 10001 and 10092 have low CD4 counts, viral loads and LAM ELISA results in addition to being single reactors to Rifampicin. Patients 10015, 10100 and 10104 have very high CD4 counts, HIV viral loads and LAM ELISA results with patient 10015 being a multiple reactor and patients 10100 and 10104 single reactors to Isoniazid and Pyrazinamide respectively.

Patients 10051 and 10085 was excluded as there was no recovery sample acquired. Patients 10001 and 10071 were analyzed in further detail in Analysis 3 as they have multiple longitudinal samples at different recovery time-points. The sample numbers included in this analysis are the following:

Table 3.8. Patient numbers corresponding to the sampling number and reaction type for analysis 2

Patient Number	Sample type	
	Acute (range of days from onset)	Recovery (Range of days into recovery)
10001	1 (28)	5 (730)
10092	9 (8)	11 (78)
10015	12 (8)	14 (90)
10100	25 (58)	26 (330)
10104	27 (9)	29 (240)

As seen in Figure 3.14 below, the sPLS-DA separates the two conditions significantly highlighting the different pathways and proteins at play within the two sample groups. The feature plot to the right of the cluster plot depicts the proteins that contribute heavily to the separation of the two clusters. It is therefore these specific proteins that are relevant to potential mechanisms and pathways important to DRESS disease resolution. On admission, the offending drugs have been stopped however, there may still be immune worsening taking place.

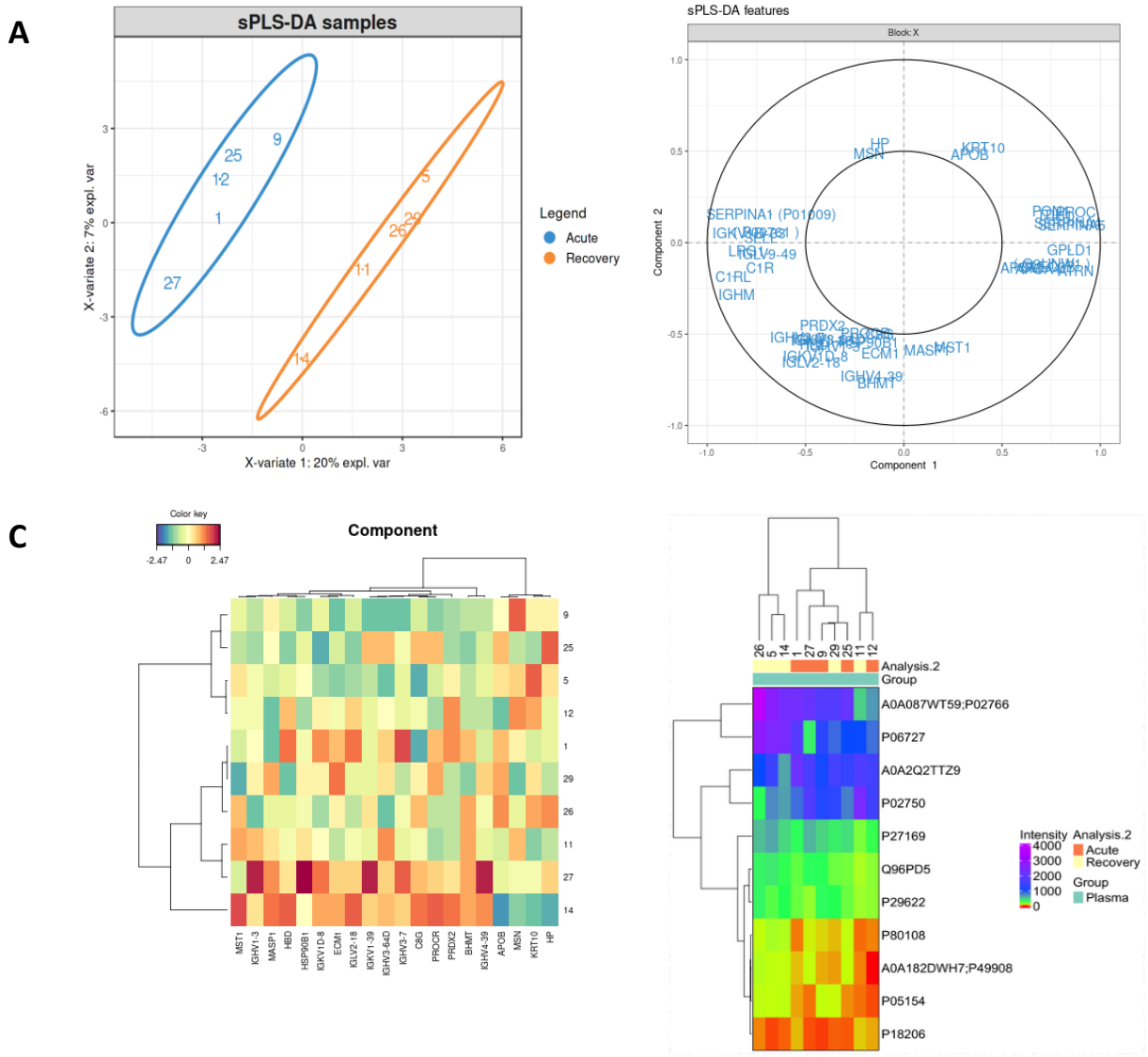


Figure 3.14: Acute HIV/TB-associated DRESS analysis *(A). sPLS-DA feature plots showing unsupervised clustering of the two, separate conditions (B). a circular sPLS-DA plot showing the top 20 genes contributing to the separation of the clusters seen in (a), (C) Heatmap showing the protein expression levels of the top 20 differentiated proteins in acute and recovery samples and, (D) ANOVA heatmap showing intensity levels of the most differentiated proteins*

Moreover, to look at these proteins in further detail, a heatmap was created. This heatmap includes the 10 samples in question and their corresponding expression of the top 20 differentially expressed proteins. The top 6 horizontal rows represent the 6 recovery samples, while the bottom 6 horizontal rows represent the 6 acute samples. The proteins that are highly expressed in the acute samples are downregulated in the recovery samples. Likewise, the proteins that are highly expressed in the recovery samples are subsequently downregulated in the acute samples. Interestingly, of the top 20 proteins that are in question, approximately half of the genes are upregulated versus downregulated in each condition. Furthermore, this

pattern of up and downregulated genes is seen across all patients regardless of the drug they reacted to.

While it is useful to outline the proteins at play, quantifying this differential expression of these proteins in question will bring light to what extent these proteins, and pathways, are operating. The fold increase/decrease values are provided next to the protein. The proteins that are upregulated for the acute sample group include the following: (P00761): Trypsin precursor ($p = 0.05$, FC = 1.8), (ABR1): Ethylene-responsive transcription factor ABR1 ($p = 0.07$, FC = 1.1), (SERPINA1): Alpha-1-antitrypsin ($p = 0.05$, FC = 1.5), (C1R) Complement subcomponent C1r ($p = 0.05$, FC = 1.2), (SELL) L-selectin ($p = 0.04$, FC = 2.5), (C1RL) Complement C1r subcomponent-like protein ($p = 0.02$, FC = 1.4), (LRG1) Leucine-rich alpha-2-glycoprotein ($p= 0.03$, FC = 2.3), and (IGKV1D-33) Immunoglobulin kappa variable 1D-33 ($p = 0.03$, FC = 1.6). These proteins are conversely downregulated in the recovery. While these proteins do function in multiple, varying pathways, there are certain pathways in common. These include the complement pathway, leukocyte-mediated immunity, and the innate immune response.

The proteins that are downregulated in the acute sample group, and upregulated in the recovery samples, include (APOM) Apolipoprotein M ($p = 0.05$, FC = -1.5), (APOA4) Apolipoprotein A-IV ($p = 0.05$, FC = -2.1), (ITIH1) Inter-alpha-trypsin inhibitor heavy chain H1 ($p = 0.06$, FC = -1.3), (CLEC3B) Tetranectin ($p = 0.04$, FC = -1.2), (PON1) Serum paraoxonase/arylesterase 1 ($p = 0.07$, FC = -1.7), (MINPP1) Multiple inositol polyphosphate phosphatase 1 ($p = 0.03$, FC=-1.7), (PROC) Vitamin K-dependent protein C ($p = 0.02$, FC = -1.5), (ATRN) Attractin ($p = 0.002$, FC = -1.3), (SERPINA4) Kallistatin ($p = 0.04$, FC = -1.6), (GPLD1) Phosphatidylinositol-glycan-specific phospholipase D ($p = 0.007$, FC = -2.2), and (SERPINA5) Plasma serine protease inhibitor ($p = 0.03$, FC = -1.9). The main pathway that these proteins function in is regulation of plasma lipoprotein oxidation.

Using a simple T test with no multiple testing correction and a fold change cut-off of 1.5, 11 proteins are differentially expressed. The ANOVA heatmap (**Figure 3.14D**) shows the intensity levels for the two sample groups in question. This does not provide a quantification but rather an indication of expression level. There is broad clustering of the two groups, as seen in the orange and yellow blocks on top of the heatmap. It is not a clean clustering; however, the samples are broadly associating with one another. There is a noticeable change in intensity level between acute and recovery samples for proteins (GPLD1) Phosphatidylinositol-glycan-specific phospholipase D, (SELENOP) Selenop P protein and (SERPINA5) Plasma serine protease inhibitor. These proteins function in lipid metabolism, oxidative stress, and anticoagulation. This further validates what is seen in the volcano plot (**figure 3.15 A**).

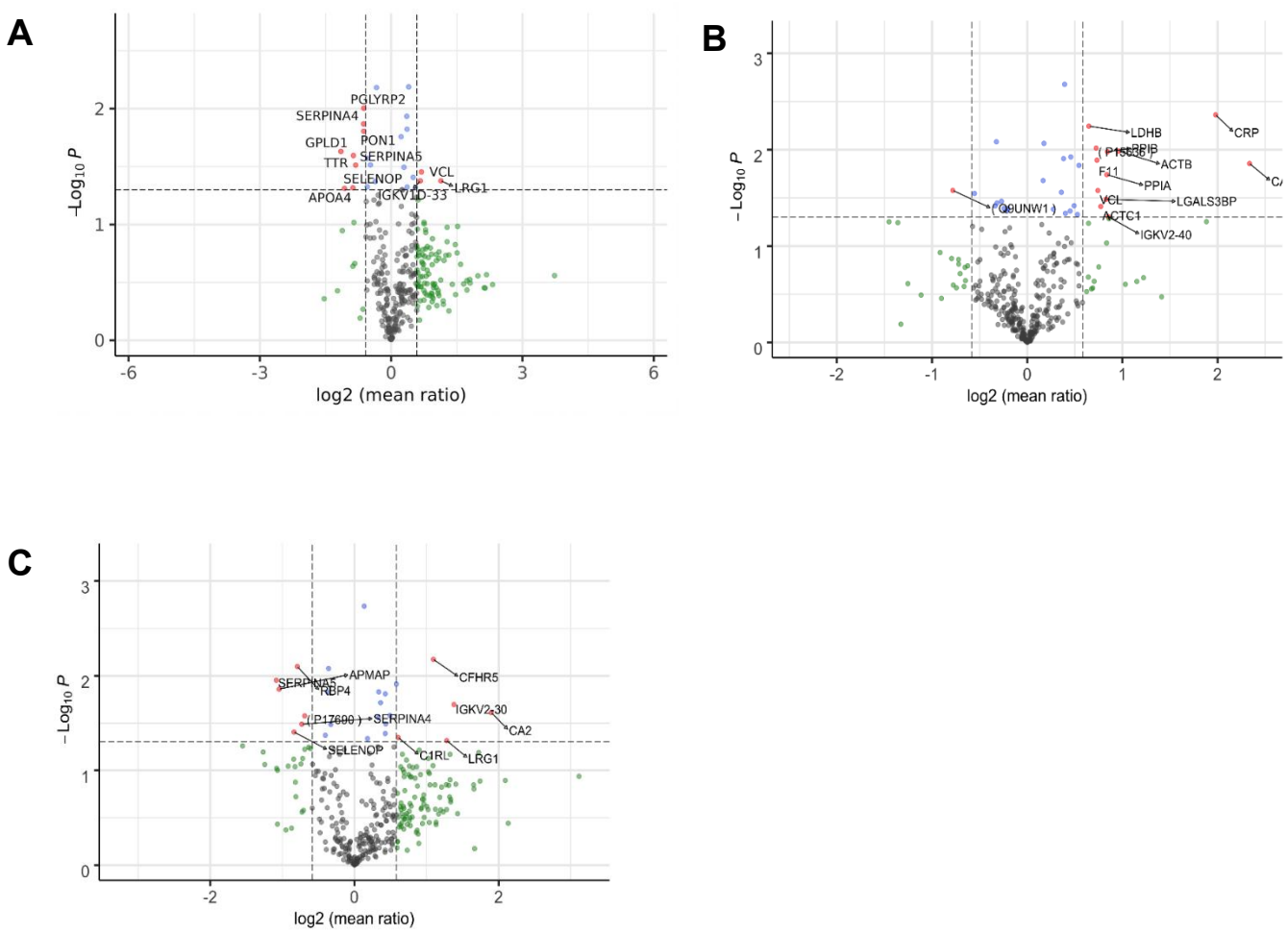


Figure 3.15: Volcano plots showing the differentially regulated proteins in the acute versus recovery sample groups. (*A) Acute vs recovery, (B) Acute vs Early recovery, (C) Acute vs Late Recovery*

Proteins to the right are upregulated while proteins to the left are downregulated. Multiple testing correction was unable to be done due to too few samples and the fold change cut off for the T test is 1.5. The y axis is the negative log of the p value (usually base 10) which results in highly significant points with low p values appearing toward the top of the plot. The log of the fold change (FC) between the two conditions is plotted on the x axis. The log of the fold

change is adopted so that changes in both directions become equidistant from the centre. Plotting points in this way form two regions of interest in the plot: those points that are found toward the top of the plot that are far too either the left- or right-hand sides. These represent values that display large magnitude fold changes as well as high statistical significance. The volcano plot on the left-hand side represents the proteins while the right-hand side volcano plot represents proteins. Upregulated proteins, as depicted by dots to the right, represents more highly abundant in the acute samples.

Volcano plots were then created to determine the differential regulation of proteins between the sample groups in question, namely Acute vs recovery, Acute vs Early recovery and Acute vs Late Recovery (**Figure 3.15 A, B and C** respectively). The three upregulated proteins in the acute samples (downregulated in the recovery samples) include (IGKV1D-33) Immunoglobulin kappa variable 1D-33 ($p = 0.03$, $FC = 1.6$), (LRG1) Leucine-rich alpha-2-glycoprotein ($p = 0.03$, $FC = 2.3$) and (VCL) Vinculin ($p = 0.09$, $FC = 1.5$) which function in the innate immune response, angiogenesis and cell adhesion and structure respectively. The proteins that are downregulated in the acute samples (upregulated in recovery samples) include (APOA4) Apolipoprotein A-IV ($p = 0.05$, $FC = -2.2$), (TTR) Transthyretin ($p = 0.1$, $FC = -2.0$), (GPLD1) Phosphatidylinositol-glycan-specific phospholipase D ($p = 0.007$, $FC = -2.2$), (PON1) Serum paraoxonase/arylesterase 1 ($p = 0.07$, $FC = -1.7$), (SERPINA4) Kallistatin ($p = 0.03$, $FC = -1.6$), (amiD) N-acetylmuramoyl-L-alanine amidase ($p = 0.08$, $FC = -1.6$), (SERPINA5) Plasma serine protease inhibitor ($p = 0.03$, $FC = -1.9$) and (SELENOP) Selenoprotein P ($p = 0.1$, $FC = -2.2$). The two proteins to note here are ApolipoproteinA-IV and Selenoprotein P. ApolipoproteinA-IV has antioxidant properties while Selenoprotein P responsible for some of the extracellular antioxidant defence properties of Selenium or might be involved in the transport of Selenium.

› Acute versus early recovery

To further our knowledge and understanding of these samples, comparison of plasma immune proteome between acute samples and both early and late recovery samples has been done. This is to investigate if there is a progression between earlier recovery and later recovery. In order to do this, acute samples were compared to early recovery samples as well as late recovery samples.

Table 3.9. Patient numbers corresponding to the sampling number and reaction type

Patient Number	Sample type	
	Acute (range of days from onset)	Recovery (Range of days into recovery)
10001	1 (28)	4 (84)
10092	9 (8)	11 (78)
10015	12 (8)	14 (90)

Clinically, these three patients all have lower CD4 counts and their acute samples that were sampled at similar time-points. However, the viral loads and LAM ELISA (TB burden) results are variable. Patient 10015 has a very high viral load and LAM ELISA result in addition to being a multiple reactor. Patients 10001 and 10092 have lower viral loads and LAM ELISA results while being single reactors to Rifampicin. The body surface area percentage affected of these three patients are relatively similar.

Figure 3.15 B shows the proteins that are upregulated in the acute versus these early recovery samples include (LDHB) L-lactate dehydrogenase B chain, (CRP) C-reactive protein, (ACTB) Actin, cytoplasmic 1, (PPIA) Peptidyl-prolyl cis-trans isomerase A, (LGALS3BP0 Galectin-3-binding protein, (IGKV2-40) Immunoglobulin kappa variable 2-40, (ACTC1) Actin, alpha cardiac muscle 1, (VCL) Vinculin, (F11) Coagulation factor XI, (PPIB) Peptidyl-prolyl cis-trans isomerase B, (P15636) Protease 1. All proteins are upregulated to a similar extent except for CRP which is upregulated to the greatest extent. These proteins function in actin organization, cell structure and morphology, innate immune response, and the complement pathway. There is only one downregulated protein which is (MINPP1) Multiple inositol polyphosphate phosphatase 1 which is a hydrolase that functions in metabolism.

› Acute vs late recovery:

Table 3.10. Patient numbers corresponding to the sampling number and reaction type

Patient Number	Sample type	
	Acute (range of days from onset)	Recovery (Range of days into recovery)
10001	1 (28)	5 (~730)
10100	25 (58)	26 (~330)
10104	27 (9)	29 (~240)

Clinically, these patients are all single reactors to Rifampicin, Isoniazid and Pyrazinamide respectively. Both patients 10100 and 10104 have high CD4 counts, viral loads and LAM ELISA results while patient 10001 has lower CD4, viral load and LAM ELISA results. Both

patients 10100 and 10104 have higher body surface area affected percentage compared to patient 10001.

The fold change was changed to less than 1.5 as no statistically significant proteins were found when the fold change was 1.5 or higher. This does necessitate further validation testing to confirm any statistically significant proteins found in this dataset using a lower fold change value. **Figure 3.15C** shows the statistically significant upregulated proteins that were found in the acute samples which included (CFHR5) Complement factor H-related protein 5, (IGKV2-30) Immunoglobulin kappa variable 2-30, (CA2) Carbonic anhydrase 2, (LRG1) Leucine-rich alpha-2-glycoprotein, and (C1RL) Complement C1r subcomponent-like protein. These proteins function in the Complement pathway, innate immune response, and metabolic processes. The downregulated proteins included (SERPINA5) Plasma serine protease inhibitor, (APMAP) Adipocyte plasma membrane-associated protein, (SERPINA4) Kallistatin, (SELENOP) Selenoprotein P, (APOH) Beta-2-glycoprotein 1, and (RBP4) Retinol-binding protein 4. These proteins function in oxidative stress, lipid metabolism and Retinol transport.

- **Analysis 3: Key protein changes across two DRESS patients with multiple samples (10001 and 10071)**

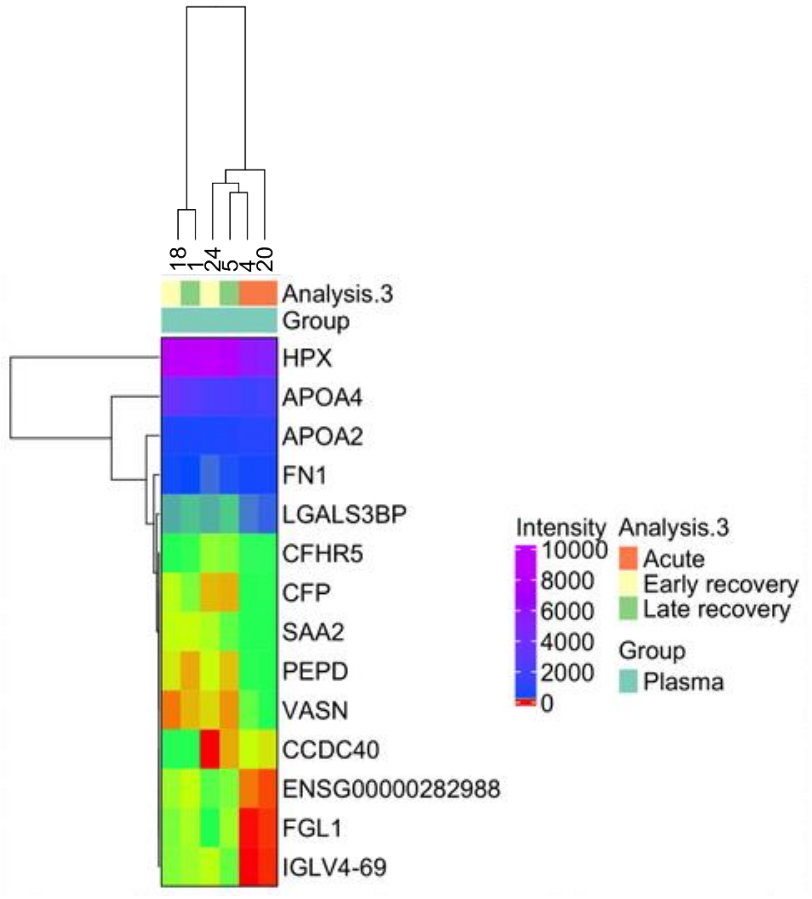
Patients 10001 and 10071 were of interest to investigate in further detail due to both their clinical and drug hypersensitivity phenotypes as well as the increased number of longitudinal samples across the disease course available. Patient 10001 reacted to Rifampicin, but a decision was made not to stop this drug and to treat through. This patient then worsened with a delayed presentation at ~3 weeks later; Rifampicin then needed to be stopped. Patient 10071 had a positive reaction to Pyrazinamide but then later tolerated this same drug during a recurrence of TB ~12 months after her initial presentation, with multiple samples taken during use of PZA in the subsequent episode of TB. Therefore, diving deeper into the immunoproteome of these two patients throughout the course of their disease may provide insight into specific pathways or proteins at play. The sample numbers included in this analysis for patient 10001 were 1 (acute sample 28 days from start of symptoms), 4 (84 days from start of symptoms) and 5 (18 months post admission with DRESS, ~730 days from start of symptoms) and for patient 10071 it was samples 18 (Acute 5 days after DRESS symptom onset, most recent CD4 330), 20 (6-months post DRESS admission ~180 days after DRESS symptom onset), 21 (~600 days from skin symptoms onset), 22 (~660 days from skin symptoms onset), 23 (~730 days from skin symptoms onset) and 24 (25-months post DRESS onset, ~750 days after skin symptoms onset), 25 (25-month post DRESS onset).

Table 3.11. Clinical details corresponding to each patient number

Patient Number	Sample type	Sample number	Days post skin symptoms	CD4+ Count	HIV Viral Load (copies/ml)	LAM ELISA (ng/ml)	Body Surface Area (%)
10001	Acute	1	28	101	40	5.3	56
	Drug reaction	2	44				
	Drug reaction delayed	3	62				
	recovery	4	84				
	18 months FU	5	730				
10071	Acute	18	5	330	262654	3.1	65
	Drug reaction PZA	19	79				

	6 months FU	20	180				
	20 months FU	21	600				
	22 months FU	22	660				
	24 months FU	23	730				
	25 months FU	24	750				

Following on from the analysis above, another heatmap was created after performing an ANOVA test using only the significant proteins (**Figure 3.16**). These heatmaps show the intensity of the expression levels of the proteins. Therefore, for the specific proteins that are shown to have differing expression levels, box and whisker diagrams are shown to quantify comparative changes over time (figure S6). Interestingly, this heatmap shows the sample clustering by timepoint or condition. Sample 1 and 18, both acute samples, cluster together while samples 4 and 20, the former an early recovery and latter a late recovery sample (very different sampling time-points), and samples 5 and 24, both early recovery (equilivant sampling time-points), cluster together. In this heatmap, there are specific proteins that are differentially expressed in each condition and should be investigated further. For example, proteins (HSD17B10) 3-hydroxyacyl-CoA dehydrogenase type-2, (VASN) Vasorin and (ACTN1) Alpha-actinin-1 are expressed in higher quantities in the acute samples than recovery samples. Moreover, proteins (FGL1) Fibrinogen-like protein 1, (IGLV4-69) Immunoglobulin lambda variable 4-69 and (PLXDC2) Plexin domain-containing protein 2 are expressed in lower quantities in acute samples than recovery samples. This pattern of expression is consistant across the two patients. Interestingly, when the expression levels are different, the major contrast that occurs in the heatmap is between acute and late recovery sample groups with early recovery expression being in the middle. This can be seen for proteins (HSD17B10) 3-hydroxyacyl-CoA dehydrogenase type-2, (ACTN1) Alpha-actinin-1 and (PLXDC2) Plexin domain-containing protein 2. This further emphasizes that there is a greater difference in protein expression levels from acute to late recovery versus acute to early recovery.


18
1
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5
4
20
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APOA2
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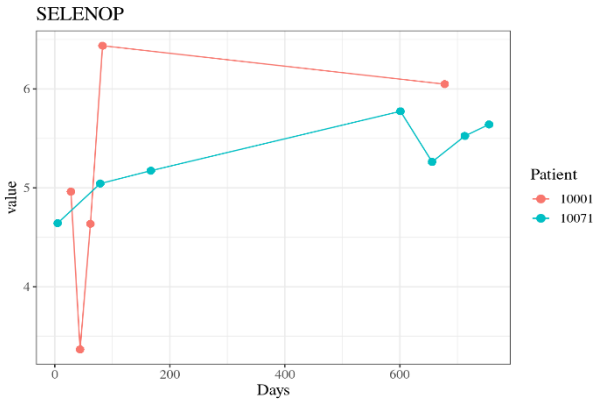
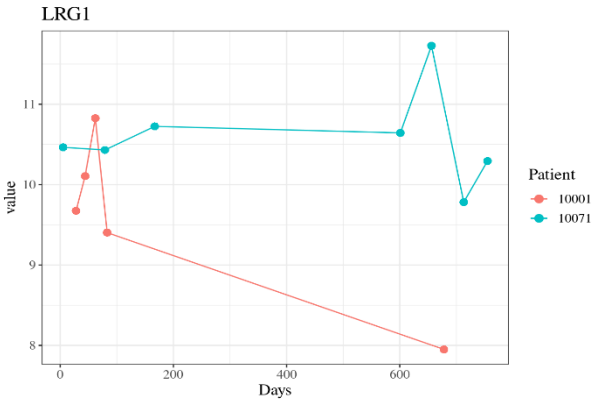
FGL1
IGLV4-69
Intensity
10000
8000
6000
4000
2000
0
Analysis.3
Acute
Early recovery
Late recovery
Group
Plasma

Figure 3.16: An ANOVA heatmap showing the differential expression of the top 20 differentially expressed proteins in this sample group. *No multiple testing correction. Fold change cut off of 1.5.*

There are multiple pathways and proteins that are expressed in patients 10001 and 10071 that were not significantly highlighted in analysis 2 when looking at the other patients in the cohort. This may be a reason behind patients 10001 and 10071 have varying clinical phenotypes and disease progression compared to other patients.


SELENOP
value
Days
Patient
10001
10071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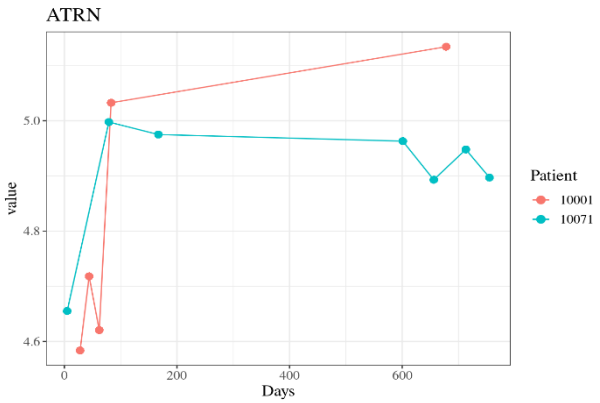
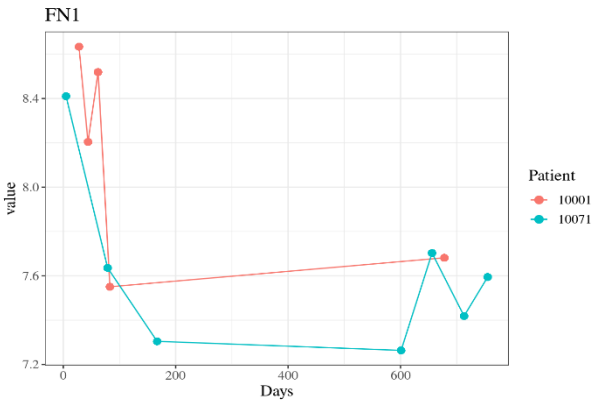
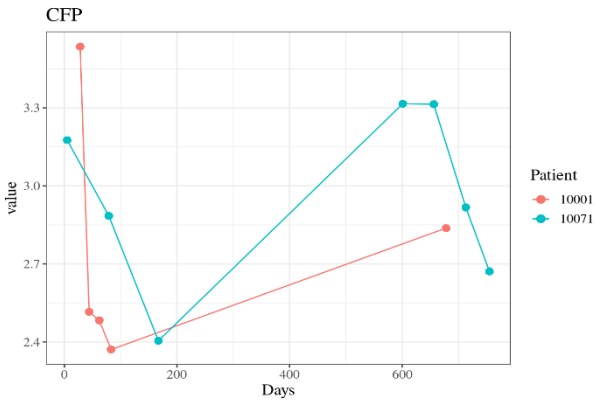
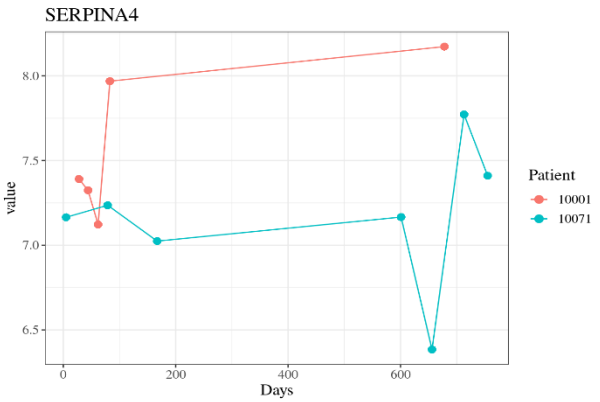
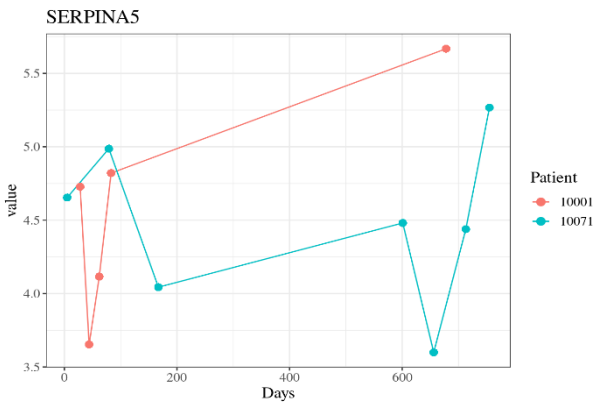
Days
Patient
10001
10071


ATRN
value
Days
Patient
10001
10071


FN1
value
Days
Patient
10001
10071


CFP
value
Days
Patient
10001
10071


SERPINA4
value
Days
Patient
10001
10071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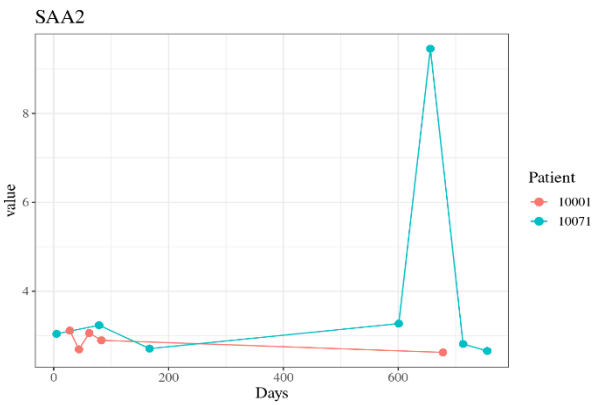
Days
Patient
10001
10071


SAA2
value
Days
Patient
10001
10071

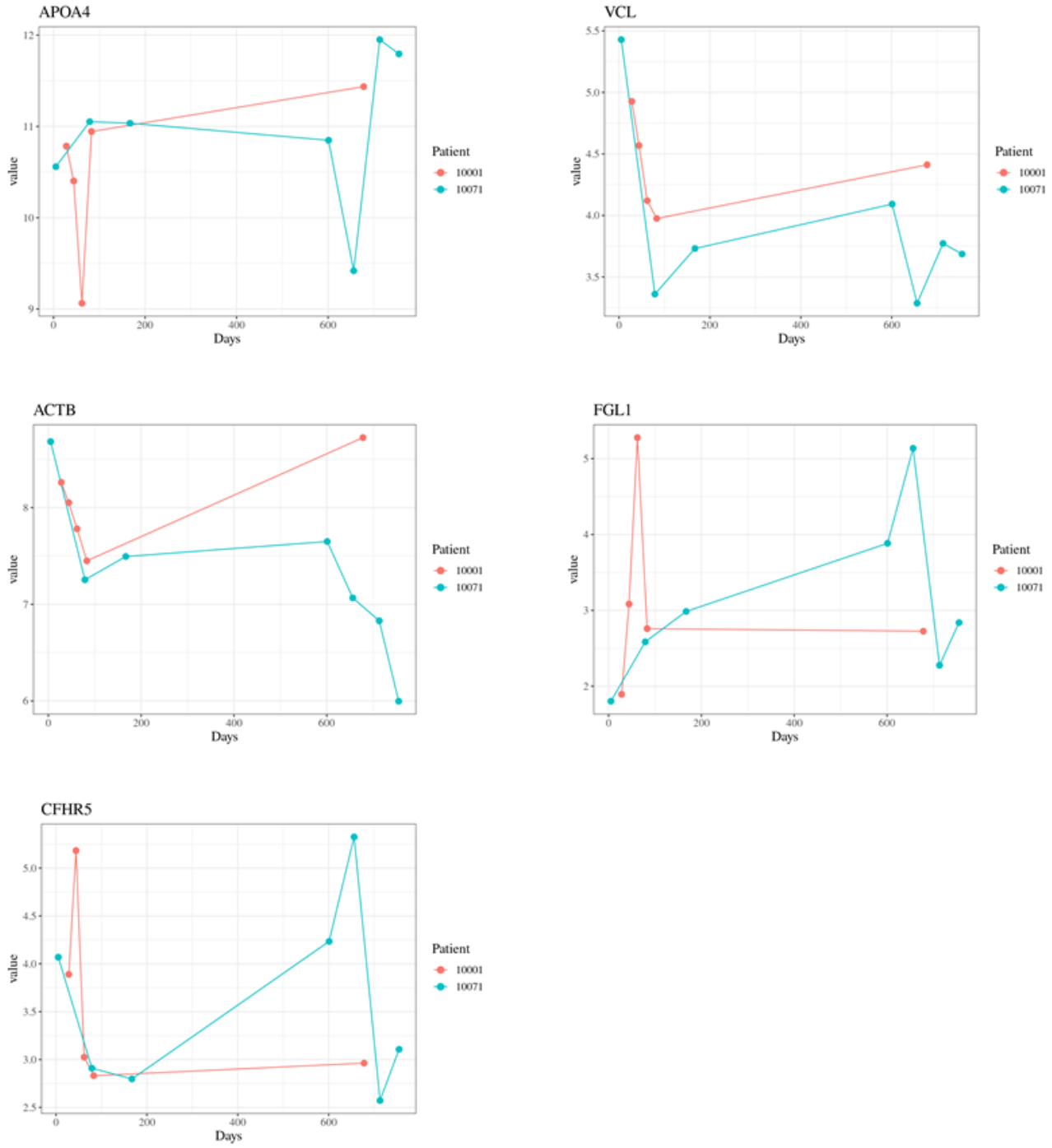


Figure 3.17: Timeline plots showing differential expression of the top differentially expressed proteins in patients 10001 and 10071. *Expression levels were measured and plotted for all samples taken for both patients. Proteins in these timeline plots include* (SELENOP) Selenoprotein P, (LRG1) Leucine-rich alpha-2-glycoprotein, (ATRN) Attractin, (FN1) Fibronectin, (CFP) Properdin, (SERPINA4) Kallistatin, (SERPINA5) Plasma serine protease inhibitor, (SAA2) Serum amyloid A-2 protein, (APOA4) Apolipoprotein A-IV, (VCL)

Vinculin, (ACTB) Actin, cytoplasmic 1, (FGL1) Fibrinogen-like protein 1 and (CFHR5) Complement factor H-related protein 5

An ANOVA test was run to compare these two patients across time points. Timeline plots were created for the top differentially regulated proteins for patients 10001 and 10071. Protein relative abundance at all sampling time points were included for this analysis. As seen in the plots, there are differences in the protein abundance between the two patients in question for majority of the highlighted proteins.

Certain proteins have a similar protein abundance pattern between both patients 10001 and 10071. Proteins that are found in higher abundance levels in the acute phase compared to drug reaction or recovery phases for both patients include (FN1) Fibronectin, (CFP) Properdin, (VCL) Vinculin. These proteins function in inducing proinflammatory cytokines, complement activation and cell adhesion and maintenance of cell shape. Conversely, proteins that are found in higher abundance levels in the drug reaction and recovery phase compared to the acute phase include (SELENOP) Selenoprotein P, and (ATRN) Attractin. These proteins function in oxidative stress and the initial immune cell clustering during inflammatory response and may regulate chemotactic activity of chemokines.

While no statistical tests can be done with only two patients due to a lack of power, it can be said that the protein abundance profiles that are different between patient 10001 and 10071 include (LRG1) Leucine-rich alpha-2-glycoprotein, (CFHR5) Complement factor H-related protein 5, (SERPINA4) Kallistatin, (SERPINA5) Plasma serine protease inhibitor, (APOA4) Apolipoprotein A-IV and (FGL1) Fibrinogen-like protein 1, (CFP) Properdin and (ACTB) Actin, cytoplasmic 1.

Patient 10001

Patient 10001 reacted to Rifampicin, but a decision was made not to stop this drug and to treat through. This patient then had a delayed reaction again ~3 weeks later. Looking into the protein expression profiles specifically for patient 10001, the proteins that had varying expression profiles around day 43 which is when this patient had a delayed reaction included 62 included (SELENOP) Selenoprotein P, (LRG1) Leucine-rich alpha-2-glycoprotein, (ATRN) Attractin,

(FN1) Fibronectin, (CFP) Properdin, (SERPINA4) Kallistatin, (SERPINA5) Plasma serine protease inhibitor, (APOA4) Apolipoprotein A-IV and (FGL1) Fibrinogen-like protein 1 and (CFHR5) Complement factor H-related protein 5.

Patient 10071

Patient 10071 had a positive reaction to Pyrazinamide but then later tolerated this same drug, with multiple samples taken during use of PZA during a subsequent episode of TB. This patient reacted to Pyrazinamide and then tolerated this drug when rechallenged a second time. Assessing the protein expression profiles above, proteins that had varying expression between the reaction of pyrazinamide (day 78) and when this drug was later tolerated (day ~860) included (FN1) Fibronectin, (CFP) Properdin, (SERPINA4) Kallistatin, (SERPINA5) Plasma serine protease inhibitor, (APOA4) Apolipoprotein A-IV, (SAA2) Serum amyloid A-2 protein, (VCL) Vinculin, (ACTB) Actin, cytoplasmic 1, (FGL1) Fibrinogen-like protein 1 and (CFHR5) Complement factor H-related protein 5.

The physiological functioning behind these proteins may contribute to the variation in the clinical features of these two patients. While there needs to be more analysis into the molecular association of these proteins to the tolerance of drugs or secondary reactions, the variation of these proteins during the specific timepoints does provide a preliminary link to change in clinical features of these patients compared to other patients.

- **Analysis 4: Single and Multiple Drug positive reaction analysis**

A key objective of this project was to have a window into the biological and molecular mechanisms at play during positive drug reactions, and to determine if differences could be noted between different clinical phenotypes of positive drug reactions e.g., fever at 24 hours versus worsening rash and ALT at 6 days. In addition, examining for differences in how the immunoproteome changed in patients having positive reactions to multiple versus only one particular anti-TB drug. This would then aid in finding a potential biomarker which could help future patients having similar phenotypes of positive drug reactions. Therefore, this analysis explores the different reactions at play and compares the reactions to the pre-challenge acute samples. Firstly, all acute samples are compared against all reaction samples to assess whether there are any highlighted proteins to begin with. Secondly, the acute samples from patients 10001, 10085 and 10104 will be compared against their positive reaction samples. These reaction samples are known as reaction 1 as they react to a single drug. Lastly, the acute samples from patients 10015 and 10051 will be compared against their positive reaction samples. These reactions form part of reaction 2 as these patients reacted to more than one of the anti-TB drugs.

***Table 3.12:* Patient numbers corresponding to the sampling number and reaction type for analysis4**

Patient Number		**Sample type**	
	Drug reaction	**Acute (days from onset of skin symptoms)**	**Reaction 1/2**
10001	2 (24 hours RF)	1 (28)	1
	3 (D15 +ve RF)		
10085	7 (D6 +ve RF)	6 (11)	
	8 (D6 +ve RB)		
10104	28 (24 hours PZA)	27 (9)	
10015	13 (D2 INH)	12 (8)	2
10051	16 (24 hours PZA, sampled D3 +ve)	15 (10)	
	17 (24 hours ETH)		

Clinically, reaction 1 patients (excluding patient 10104) have lower CD4 counts, LAM ELISA results and both reacting to Rifampicin. Patient 10104 has high CD4 counts, high HIV viral load and a high LAM ELISA. Moreover, this patient reacted to Pyrazinamide. These two patients have the same body surface area percentages. Reaction 2 patients have variable clinical

phenotypes with patient 10015 having higher CD4 count, viral load, ALT and LAM ELISA results but much lower eosinophil count compared to patient 10051. The body surface area affected is also variable with 30% and 60% for patients 10015 and 10051 respectively.

Before diving deeper into the specific analyses of reaction 1 and 2, the overall reaction was investigated. This was done by comparing all acute samples and all reaction samples. The two sample groups cluster separately with no overlap in the sPLS-DA plot emphasizing the fact that there are differentially regulated proteins at play in the two groups. This clustering was a supervised clustering meaning that the analysis is done with no regard to the condition or grouping of the samples. **Figure 3.18B** is a PCA (principal component analysis) shows an unsupervised approach of clustering. There is a large overlap in these two sample groups thereby meaning that when there is no supervised clustering, these samples appear to not have major protein differentiation resulting in the overlap of the clusters seen.

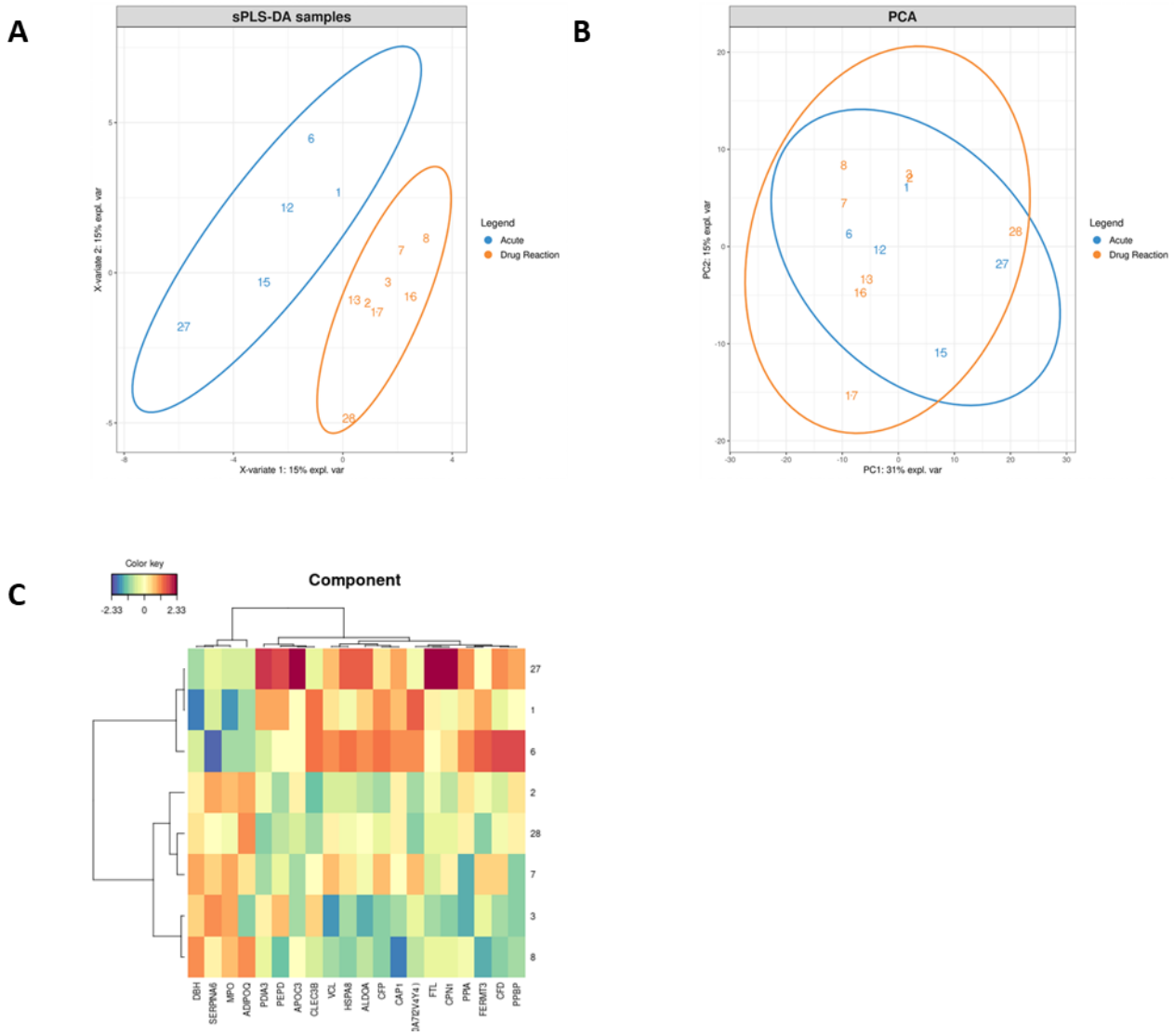


Figure 3.18: Acute versus positive reaction sample analysis *(A). sPLS-DA plot showing the supervised clustering of the two sample groups, acute and reaction samples, (B) PCA plot showing of the unsupervised clustering of these two sample groups, (C) Heatmap showing the protein expression levels of the acute and drug reaction samples for the top 20 differentiated proteins*

There is a broad separation of clusters evident in the heatmap. Firstly, the top 3 and the bottom 5 samples cluster together with up- and downregulation ($q < 0.05$, FC = 1.5) seen in both clusters. There is a pattern of regulation in which the proteins corresponding to top right and bottom left clusters are upregulated while proteins in the top left and bottom right are

downregulated. There is one distinct factor that can be noted, samples 27, 1 and 6 (similar sampling times) are all acute samples. Samples 7, 3 and 8 (similar sampling times) also have a similar profile and are all drug positive reaction samples (delayed reactions to Rifampicin). Lastly, samples 2 and 28, which cluster next to each other, are drug positive reactions at 24 hours and 2 days. The proteins that are upregulated in samples 27, 1 and 6 in the heatmap include (ADIPOQ) Adiponectin, (PDIA3) Protein disulfide-isomerase A3, (PEPD) Xaa-Pro dipeptidase, (APOC3) Apolipoprotein C-III, (CLEC38) C-type Lectin, (VCL) Viculin, (HSPA8) Heat shock cognate 71 kDa protein, (ALDOA) Fructose-bisphosphate aldolase A, (CFP) Properdin, (CAP1) Adenylyl cyclase-associated protein 1, (FTL) Ferritin light chain, (CPN1) Carboxypeptidase N catalytic chain, (PPIA) Peptidyl-prolyl cis-trans isomerase A, (FERMT3) Fermitin family homolog 3, (CFD) Complement factor D and (PPBP) Platelet basic protein. These proteins are downregulated in the drug positive reaction samples. These proteins function in complement activation, neutrophil degranulation, leukocyte activation involved in the immune response and platelet degranulation. The proteins that are downregulated in the acute samples (and upregulated in the drug positive reaction samples) include (DBH) Dopamine beta-hydroxylase, (SERPINA6) Corticosteroid-binding globulin and (MPO) Myeloperoxidase. These three proteins do not have any major pathway overlap but overall, they function in Conversion of dopamine to noradrenaline, transport protein for glucocorticoids and progestins in the blood and the hosts defense system by enhancing antimicrobial activity.

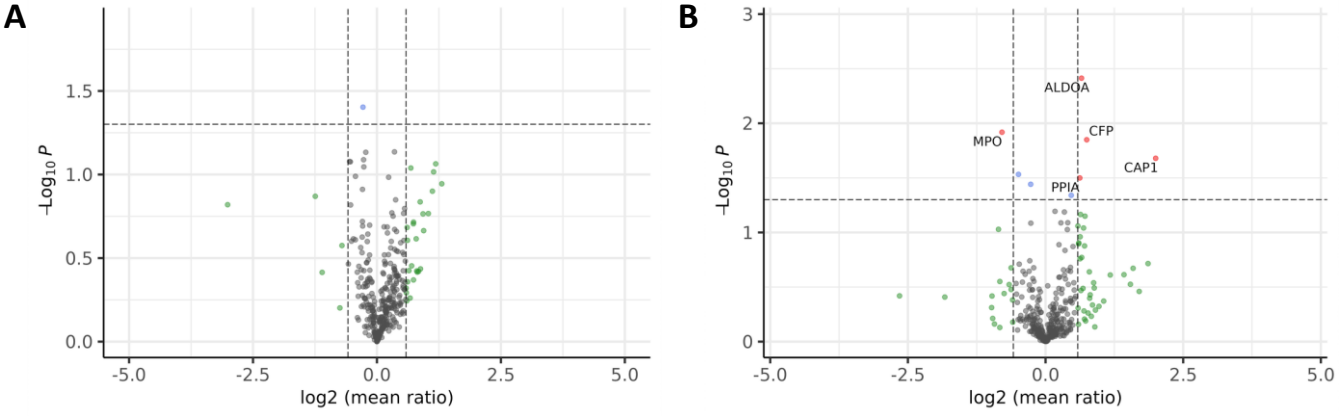


Figure 3.19: Volcano plots showing the most differentially expressed proteins when comparing acute versus reaction samples. *Fold change cut off = 1.5, no MTC applied. (A) Acute vs drug reaction, (B) Acute vs reaction 1, (C) Acute vs reaction 2 and (D) Reaction 1 vs reaction 2.*

Volcano plots were then created to assess the differential regulation of proteins between the following groups: Acute vs drug reaction, Acute vs reaction 1, Acute vs reaction 2 and Reaction 1 vs reaction 2 (**Figure 3.19**). After performing a T Test, with a fold change of 1.5 and no multiple testing correction due to insufficient power, there were no significant proteins were highlighted between these two sample groups (acute and drug reaction). This coincides with the PCA plot (**Figure 3.18B**) in which the unsupervised clustering of these samples was overlapping and not separated out thereby showing that there is little differentiation between

the sample groups. Therefore, it was important to separate out the drug reaction samples in order to assess the differential protein expression in a greater level of granularity.

Acute versus reaction 1:

Going one step deeper, Reaction 1 samples were then used in an analysis to further characterize these reactions which may, in turn, expand our understanding of what is driving DRESS pathogenesis or underpinning n on-specific flare up reactions. To reiterate, the reaction 1 includes samples from patients who reacted to only one drug. In this case, patients 10001 and 10085 who both reacted to Rifampicin and patient 10104 who reacted to Pyrazinamide.

Figure 3.19B shows that the following proteins: (ALDOA) Fructose-bisphosphate aldolase A ($p = 0.004$, FC = 1.6), (MPO) Myeloperoxidase ($p = 0.04$, FC = 1.4), (PPIA) Peptidyl-prolyl cis-trans isomerase A ($p = 0.03$, FC = 1.6), (CFP) Properdin ($p = 0.014$, FC = 1.7) and (CAP1) Adenylyl cyclase-associated protein 1 ($p = 0.02$, FC = 4) where differentially regulated. MPO is the only protein downregulated in acute samples versus reaction 1 samples with the other four proteins (ALDOA, PPIA, CFP and CAP1) being upregulated in acute and downregulated in reaction 1 samples. MPO, Myeloperoxidase, has an antimicrobial activity and is involved in the hosts defence system. It is interesting this protein is upregulated in reaction 1 samples (downregulated in acute samples). ALDOA, Fructose-bisphosphate aldolase A, functions in glycolysis and gluconeogenesis. PPIA, Peptidyl-prolyl cis-trans isomerase A, plays a role in multiple pathways including the proinflammatory pathway, oxidative-stress induced apoptosis, viral replication inhibition and exerts a strong chemotactic effect on leukocytes. CFP, Properdin, functions in the complement pathway. Lastly, CAP1, Adenylyl cyclase-associated protein 1, regulates filament dynamics and is involved in a number of complex developmental and morphological processes. Therefore, it can be stated that proteins involved in the innate immune response, morphological maintenance and oxidative stress are upregulated in reaction 1 samples compared to acute samples.

Acute versus reaction 2:

Following on from the previous analysis, reaction 2 samples were then used for further investigation to understand if there are any key proteins/pathways highlighted here that are different to reaction 1. Again, to reiterate, reaction 2 samples are from patients who reacted to more than one drug.

In the volcano plot (**Figure 3.19C**), there are ten significantly differentiated proteins identified. There are four proteins that are downregulated in acute samples (upregulated in reaction 2 samples) while six proteins are upregulated in acute samples (downregulated in reaction 2 samples). (GLPD1) Phosphatidylinositol-glycan-specific phospholipase D (p = 0.004, FC = 1.6), (RBP4) Retinol-binding protein 4 (p = 0.01, FC = -2.2), (TTR) Transthyretin (p = 0.01, FC = -2.7), (SERPINA4) Kallistatin (p = 0.04, FC = -1.7) are downregulated in acute samples. These proteins function in mediating retinol transport in blood plasma, transports thyroxine from the bloodstream to the brain. (IGHD) Immunoglobulin heavy constant delta (p = 0.005, FC = 1.8), (IGKV3-7) Probable non-functional immunoglobulin kappa variable 3-7 (p = 0.03, FC = 2.0), (IGLV4-69) Immunoglobulin lambda variable 4-69 (p = 0.03, FC = 2.1), (LYST) Lysosomal-trafficking regulator (p = 0.03, FC = 2.7), (SERPINA3) Alpha-1-antichymotrypsin (p = 0.04, FC = 1.5) and (CRP) C-Reactive protein (p = 0.04, FC = 5.7) are downregulated in reaction 2 samples. These proteins function in the innate immune response and the production of pro-inflammatory cytokines.

Reaction 1 vs reaction 2

Lastly, in order to determine the differences that are apparent in patients that react to a single drug versus multiple drugs, reaction 1 samples are compared to reaction 2 to identify differentially regulated proteins at play. As noted in the volcano plot (**Figure 3.19D**), there are 23 differentially regulated proteins within these two sample groups.

There are 9 downregulated proteins in reaction 1 samples versus reaction 2 including (GPLD1) Phosphatidylinositol-glycan-specific phospholipase D (p = 0.0004, FC = -2.5), (IGHD) Immunoglobulin heavy constant delta (p = 0.004, FC = -2.5), (TF) Serotransferrin (p = 0.01, FC = -2.1), (LGALS3BP) Galectin-3-binding protein (p = 0.02, FC = -2.2), (PF4) Platelet factor 4 (p = 0.03, FC = -2.4), (PRDX6) Peroxiredoxin-6 (p = 0.03, FC = -4.0), (CAP1) Adenylyl cyclase-associated protein 1 (p = 0.04, FC = -5.3), (PCYOX1) Prenylcysteine oxidase 1 (p = 0.03, FC = -2.3), and (MBL2) Mannose-binding protein C (p = 0.04, FC = -1.6). Interestingly, these proteins do not have many functioning's or pathways in common with one another. Instead, they individually function in a variety of pathways including the degradation of prenylated proteins, cell protection against oxidative stress, innate immune defence, integrin-mediated cell adhesion, stimulate host defence against viruses, iron storage, inhibition of angiogenesis and morphological processes.

There are 14 proteins upregulated in reaction 1 (downregulated in reaction 2 samples) including (SERPINA1) Alpha-1-antitrypsin (p = 0.3, FC = -1.2), (IGLV8-61) Immunoglobulin lambda variable 8-61 (p = 0.01, FC = 3.2), (IGLV4-69) Immunoglobulin lambda variable 4-69 (p = 0.04, FC = 1.8), (IGHG3) Immunoglobulin heavy constant gamma 3 (p = 0.01, FC = 4.4), (IGLV1-51) Immunoglobulin lambda variable 1-51 (p = 0.01, FC = 5.3), (HSD17B10) 3-hydroxyacyl-CoA dehydrogenase type-2 (p = 0.02, FC = 7.8), (LYST) Lysosomal-trafficking regulator (p = 0.04, FC = 2.7), (CRP) C-reactive protein (p = 0.04, FC = 5.2), (LUM) Lumican (p = 0.04, FC = 2.0), (IGLV3-9) Immunoglobulin lambda variable 3-9 (p = 0.04, FC = 4.6), (C2) Complement C2 (p = 0.2, FC = 1.2), (FBLN1) Fibulin-1 (p = 0.03, FC = 1.6), (C1QB) Complement C1q subcomponent subunit B (p = 0.04, FC = 1.9), and (IGKV1-8) Immunoglobulin kappa variable 1-8 (p = 0.05, FC = 2.5). These proteins are heavily involved in the innate immune response, complement activation, leukocyte-mediated immunity (C2, C1QB, CRP, SERPINA1 and LYST) and lymphocyte mediated immunity (C2, C1QB, CRP and LYST). Moreover, these proteins also function in cell adhesion and sorting endosomal resident proteins into late multivesicular endosomes by a mechanism involving microtubules.

Comparing the proteins that are upregulated in reaction 1 versus proteins that are upregulated in reaction 2 could provide a preliminary link to the differentiation of single versus multiple drug reacting patients. This could lead to biomarker identification and pathway analysis of single versus multiple drug reactions. The proteins upregulated in reaction 1 function in the innate immune response, cell adhesion, complement activation and lymphocyte mediated immunity. The proteins upregulated in reaction 2 samples function in cell protection against oxidative stress, innate immune defence, integrin-mediated cell adhesion, stimulate host defence against viruses, iron storage, inhibition of angiogenesis and morphological processes. Therefore, there are different pathways at play in reaction 1 and reaction 2 samples which may explain why certain patients react to a single drug versus multiple drugs. More specifically, lymphocyte mediated immunity and innate immunity/complement activation might be a reason that patients react to multiple drugs while host defence against viruses, iron storage and inhibition of angiogenesis may result in patients reacting to single drugs and not multiple drugs.

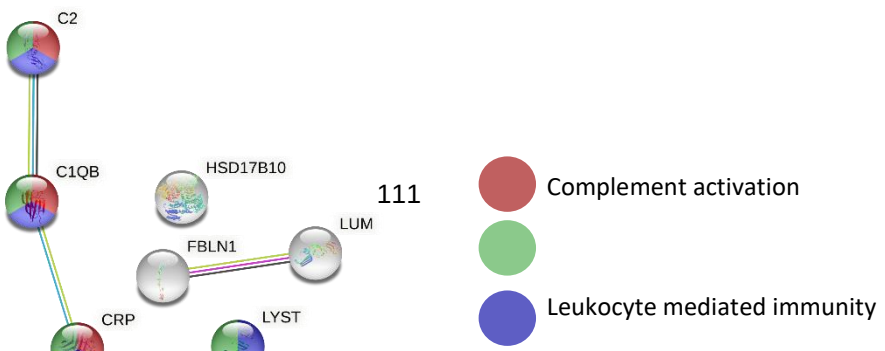


Figure 3.20: STRING figure showing the connectedness of the proteins in question for the reaction 1 vs 2 analysis*. Proteins in this figure include: (C2) Complement C2, (C1QB) Complement C1q subcomponent subunit B, (CRP) C-Reactive protein, (SERPINA1) Alpha-1-antitrypsin, (HSD17B10)) 3-hydroxyacyl-CoA dehydrogenase type-2, (FBLN1) Fibulin-1, (LYST) Lysosomal-trafficking regulator and (LUM) Lysosomal-trafficking regulator. Confidence cut off of 0.5.*

The proteins upregulated in reaction 2 samples function in leukocyte mediated immunity (C2, C1QB, CRP, SERPINA1 and LYST), lymphocyte mediated immunity (C2, C1QB, CRP and LYST) and complement activation (C2, C1QB and CRP). As seen in the STRING figure above, these proteins are involved in pathways that are interconnected. Namely, C2, C1QB, CRP and SERPINA1 function in the pathways above and these proteins are connected in their functioning.

- **Landscape summary of results:**

Below is a landscape of the overall differentially regulated proteins in the recovery analysis and drug positive reaction analysis. The proteins that are upregulated in the acute (for example) are downregulated in the recovery (for example) and vica versa. These tables were crucial to provide an overview of all proteins highlighted in the analysis to look at the sample comparisons from a holistic view.

Table 3.13: Summary of all significantly differentially regulated proteins in the Acute vs Recovery analysis

	Acute (vs recovery)	**Acute (vs early recovery)**	**Recovery (versus acute)**
Upregulated proteins	Trypsin precursor (ABR1) Ethylene-responsive transcription factor (SERPINA1) Alpha-1-antitrypsin (C1R) Complement subcomponent C1r (SELL) L-selectin (CRL1) Complement C1r subcomponent-like protein (LRG1) Leucine-rich alpha-2-glycoprotein (IGKV1D-33) Immunoglobulin kappa variable 1D-33	(LDHB) L-lactate dehydrogenase B chain (CRP) C-reactive protein (ACTB) Actin, cytoplasmic 1 (PPIA) Peptidyl-prolyl cis-trans isomerase A (LGALS3BP) Galectin-3-binding protein (IGKV2-40) Immunoglobulin kappa variable 2-40 (ACTC1) Actin, alpha cardiac muscle 1, (VCL) Vinculin (F11) Coagulation factor XI (PPIB) Peptidyl-prolyl cis-trans isomerase B Protease 1	(APOM) Apolipoprotein M (APOA4) Apolipoprotein A-IV (ITIH1) Inter-alpha-trypsin inhibitor heavy chain H1 (CLEC3B) Tetranectin (PON1) Serum paraoxonase/arylesterase 1 (MINPP1) Multiple inositol polyphosphate phosphatase 1 (PROC) Vitamin K-dependent protein C (ATRN) Attractin (SERPINA4) Kallistatin (GPLD1) Phosphatidylinositol-glycan-specific phospholipase D (SERPINA5) Plasma serine protease inhibitor

	(SELENOP) Selenop P protein (BHMT) Betaine--homocysteine S-methyltransferase 1 (PRG2) Bone marrow proteoglycan		

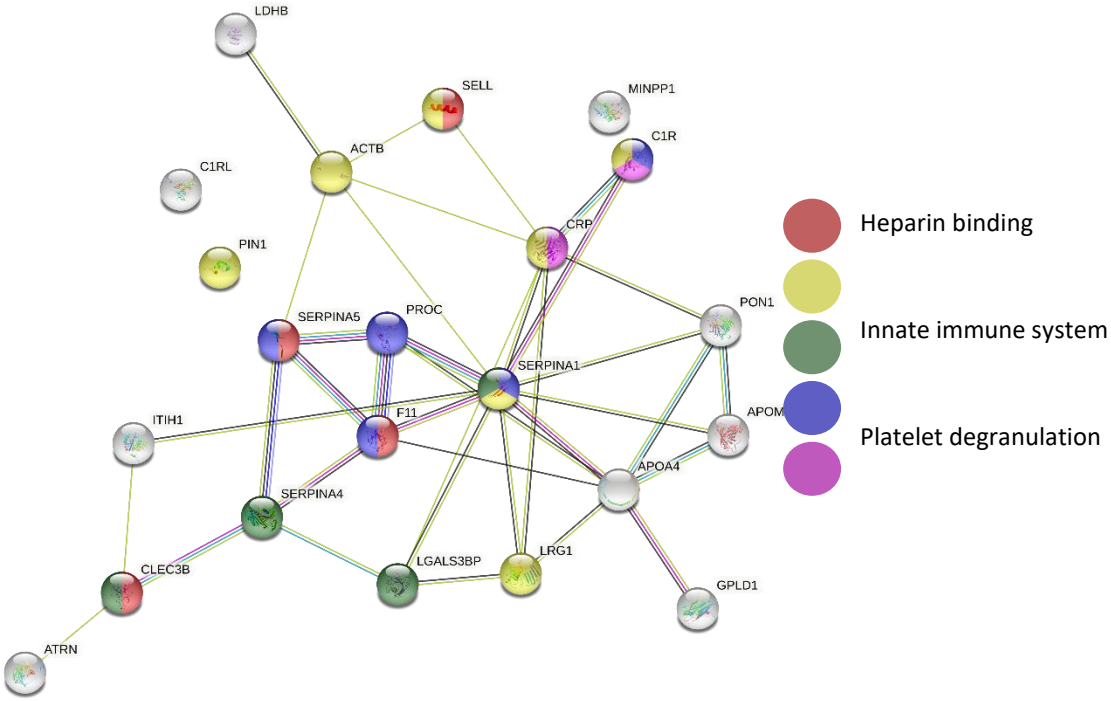


Figure 3.21: STRING figure presenting the connectedness of all the significantly differentially regulated proteins from recovery analysis. *Confidence cut off of 0.5.*

The proteins presented in the STRING figure were highlighted in the acute vs recovery and acute vs early recovery analysis. Overall, these proteins together function in three main pathways including Heparin binding, Calcium ion binding and Serine-type endopeptidase activity. SERPINA1 is at the center of the matrix of proteins in the figure. SERPINA1 is Alpha-1-antitrypsin is a serine protease inhibitor. This protein is therefore functioning with multiple other proteins in multiple pathways. Therefore, SERPINA1 can be considered an important factor in driving multiple reactions.

	Reaction 1 (vs Acute)	Reaction 2 (vs Acute)	Reaction 1 (vs Reaction 2)
Upregulated Proteins	(MPO) Myeloperoxidase	(GPLD1) Phosphatidylinositol-glycan-specific phospholipase D (RBP4) Retinol-binding protein 4 (TTR) Transthyretin (SERPINA4) Kallistatin	(SERPINA1) Alpha-1-antitrypsin (IGLV8-61) Immunoglobulin lambda variable 8-61 (IGLV4-69) Immunoglobulin lambda variable 4-69 (IGHG3) Immunoglobulin heavy constant gamma 3 (IGLV1-51) Immunoglobulin lambda variable 1-51 (HSD17B10) 3-hydroxyacyl-CoA dehydrogenase type-

			(LYST) Lysosomal-trafficking regulator (CRP) C-reactive protein LUM) Lumican (IGLV3-9) Immunoglobulin lambda variable 3-9 (C2) Complement C2 (FBLN1) Fibulin-1 (C1QB) Complement C1q subcomponent subunit B (IGKV1-8) Immunoglobulin kappa variable 1-8
Downregulated Proteins	(ALDOA) Fructose-bisphosphate aldolase A (PPIA) Peptidyl-prolyl cis-trans isomerase A (CFP) Properdin (CAP1) Adenylyl cyclase-associated protein 1	(IGHD) Immunoglobulin heavy constant delta IGKV3-7) Probable non-functional immunoglobulin kappa variable 3-7 IGLV4-69) Immunoglobulin lambda variable 4-69 (LYST) Lysosomal-trafficking regulator (SERPINA3) Alpha-1-antichymotrypsin (CRP) C-Reactive protein	(GPLD1) Phosphatidylinositol-glycan-specific phospholipase D (IGHD) Immunoglobulin heavy constant delta TF) Serotransferrin (LGALS3BP) Galectin-3-binding protein (PF4) Platelet factor 4 (PRDX6) Peroxiredoxin-6 (CAP1) Adenylyl cyclase-associated protein 1 (PCYOX1) Prenylcysteine oxidase 1

			(MBL2) Mannose-binding protein C

Table 3.14: Summary of all significantly differentially regulated proteins in the drug positive reaction analysis

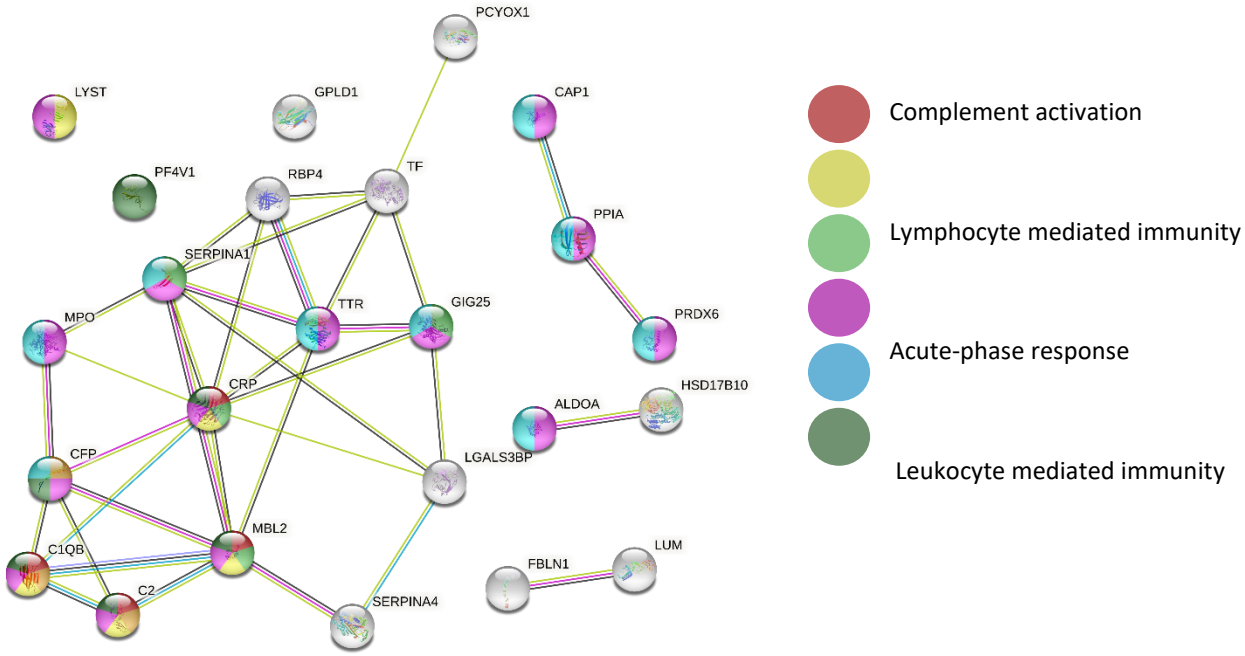


Figure 3.22: STRING figure presenting the connectedness of all the significantly differentially regulated proteins from drug positive reaction analysis. *Confidence cut off of 0.5.*

The proteins presented in the STRING figure are all proteins that are significantly differentially regulated in the drug positive reaction analysis. These proteins function in pathways including, Complement activation, Acute phase response, Regulation of complement activation, Lymphocyte mediated immunity, Leukocyte mediated immunity, Humoral immune response, and Neutrophil degranulation. This emphasizes that the proteins that are driving the changes in patients that react to a single drug versus multiple drugs function in the pathways mentioned above. Majority of the proteins that are highlighted as differentially regulated are immune-related thereby showing that the changes that occur between the group of patients in this analysis are immune-mediated.

— Discussion

Globally, DRESS and SJS/TEN are the two most common SCARs clinical phenotypes and occur up to 100-fold more frequently amongst persons living with HIV (74). South Africa has some of the highest rates of active and latent TB in the world, and 60% of new TB cases are co-infected with HIV (104). This high prevalence provides considerable prescribing pressure, and first-line anti-tuberculosis agents, cotrimoxazole (to prevent opportunistic infections) and antiretrovirals being the commonest drugs prescribed for persons living with HIV in SA, and the commonest offending agents in SCAR. SCAR in the context of HIV-related advanced immunosuppression and co-infected TB carries considerable morbidity, medical complexity, and healthcare cost (7, 16).

HIV/TB co-infected patients with SCAR secondary to anti-TB drugs undergo both the early introduction of three drug bridging therapy, as well as sequential drug challenge (SDC) to first-line anti-TB drugs to both identify rapidly the offending drug(s), and re-establish an effective multidrug TB regimen; although few grade 3 and 4 adverse events occur (7) To ensure that these vulnerable patients a) do not unnecessarily have useful drugs removed, and b) are rapidly re-established on an effective anti-TB multidrug regimen; HIV co-infected patients has resulted in these drugs being the most common offenders for SCAR. In the current study, we sought to characterize the immunoproteome of these reactions in our cohort of comorbid HIV-TB infection, with predominance of reactions to first-line anti-tuberculosis drugs, and to possibly further understand the molecular mechanisms underpinning these reactions with the hope of identifying potential biomarkers. We present here several different proteomic comparisons comparing patients, differing time-points across DRESS, and diverse positive reactions to *in vivo* oral drug rechallenge to FLTB to identify the key pathways change under different conditions as well as potential biomarkers for distinguishing different phenotypes of drug rechallenge reaction.

Plasma immune proteomes of individual DRESS patients cluster more with each other than across DRESS disease states

DRESS occurring in the context of advanced HIV-associated immunosuppression and concomitant active, and usually disseminated TB with a very high six-month mortality is a complex clinical and pathological entity (105). Consequently, every effort was made to ensure RegiSCAR validated DRESS

phenotyping was performed, and collected as much data on clinical, laboratory, and other pathogen factors that may impact the immunoproteome. This included: i) body surface area, highest liver function abnormalities and eosinophil counts as measurement of DRESS severity, ii) HIV viral activity (viral load) and immunosuppression (CD4 cell counts); ii) the serostatus and viral activity of chronic herpes viruses known to reactivate in the context of DRESS (CMV, EBV and HHV6) (106); and iii) an estimate of mycobacterial disease burden using serum LAM concentration - associate with mortality amongst this patient population (107)), together with details on anti-TB treatment duration prior to DRESS onset and use of bridging therapies.

To get an initial overview of the proteomic dataset, we included all samples looking for proteomic clusters and the major differential proteins expressed. It was encouraging to see DRESS disease conditions e.g., acute versus recovery showing distinct proteomic differences visualised in principal component analysis. However, more discriminating were the interindividual proteins differences between DRESS patients e.g., 10001 versus 10104, and the heatmap of the top 20 differentially regulated protein show patient samples clustering together irrespective of being acute versus recovery DRESS samples. These 20 differentially expressed proteins included predominantly acute phase immune proteins, several immunoglobulin fractions, and proteins involved in cell binding, motility, and morphology (Fibrinogen alpha chain, (FGB) Fibrinogen beta chain precursor, (PZP) Pregnancy zone protein, (IGHA2) Immunoglobulin heavy constant alpha 2, (IGHA1) Immunoglobulin heavy constant gamma 1, (C2) Complement C2). It is challenging to understand the relative contributions of DRESS-related inflammatory changes, as compared to other known drivers of acute and chronic inflammation such as uncontrolled HIV and active TB (108). Exploration of covariates for individual patients suggests that the likely drivers of interindividual patient differences are TB disease burden (as measured by median LAM concentration), and perhaps liver function abnormalities, although this small cohort lacks statistical power to make a clear conclusion.

Specific interest was placed on determining the interindividual differences during disease progression in the patients. Looking at the expression of all differentially regulated proteins in the samples, two proteins were identified to be changing significantly throughout the disease progression amongst all patients. Betaine--homocysteine S-methyltransferase 1 (BHMT) and C-Reactive protein (CRP) had varying expression levels (supplementary figures). BHMT is associated with acute liver injury and this protein was higher in intensity in patients that had high ALT, AST, or ALP tests (109). For example, patient 10001 AST level went from 76 – 640 during the positive reaction. This coincides with BHMT

increasing. Liver injury or damage has been found to be involved in DRESS pathogenesis and this finding provides a preliminary proteomic link. This protein has the potential to be a therapeutic target to prevent severe disease progression. CRP is a positive acute phase protein (APP) whose fold change in response to triggering cytokines is greater than several of the other APPs (110), and this proteins' levels are elevated in acute samples for several patients (10015, 10051, 10092, 10100 and 10104) but for other patients it is elevated during the reaction/disease progression (10001, 10071 and 10085). While these skin symptoms did occur during the acute phase in all patients, the elevated CRP expression in certain patients may be an indication that the skin symptoms occurred to a greater severity extent. The patients that had elevated CRP levels in the reaction samples all experienced severe positive drug reactions in which they presented with visible rashes.

HIV and TB drive acute and chronic inflammation with corresponding changes in serum proteins. Several proteomic studies of TB in persons living with HIV have demonstrated signatures related to disease activity and burden (111, 112) all of which involved acute phase proteins. C-reactive protein – a pentraxin acute phase protein produced by the liver – has been validated as a diagnostic marker for TB in persons living with HIV (113). Interestingly, this diagnostic utility is evident in the detailing of patient 10071 where one of the recovery DRESS samples show increases CRP/serum amyloid A reflective of her developing a new episode of extrapulmonary TB lymphadenitis. HIV infection is a strong driver of chronic inflammatory changes, most evident in the serum proteome by the persistent polyclonal hypergammaglobulinemia which is a known feature of HIV (as well as other viral infections and autoimmune/autoinflammatory processes) (114). This is a particularly important consideration in the light of several of our analyses that demonstrated up and downregulation of immunoglobulin heavy and light chains. The mechanisms of polyclonal hypergammaglobulinemia are not fully elucidated, but HIV-infected CD4 T-cells have been shown to induce noncognate contact-dependent polyclonal B-cell activation, with production of very high amounts of IgM, IgG, and IgA (115) and in the context of other viral infections CD4 helper T-cells have been shown to drive antibody product from B-cells that have processed viral antigens irrespective of B-cell receptor specificities (116). Inflammasome-related cytokines e.g., IL-1, and IL-6 together with TH1 cytokines (IFNg and TNFa) also appear to be important and may indicate why in the context of DRESS-induced acute inflammation different antibodies are increasing (117).

Acute DRESS to first-line anti TB treatment is characterised by dominant acute phase response with slow resolution and tissue repair

We conducted several different analyses to identify overall proteome changes occurring during acute DRESS to FLTB in persons with advanced immunosuppression, focusing on intraindividual changes more readily attributable to DRESS rather than HIV/TB related inflammatory change. Major upregulated proteins are summarised in Table 3.13 and accompanying STRING figure (figure 3.21). Acute phase proteins and immunoglobulin kappa variable chains dominate the serum proteome of acute DRESS. This is remarkable given that all samples underwent 14 protein depletion to remove immunoglobulins and several acute phase proteins including: (Alpha 1 antitrypsin, Haptoglobin, IGHA1 protein, IGHG1 protein, Serotransferrin, Complement C3, Apolipoprotein A-I and A-II, Transthyretin) (96).

The varied reactions of the host to infection, inflammation, or trauma are collectively known as the acute-phase response (APR) and encompass a wide range of pathophysiological responses such as pyrexia, leukocytosis, hormone alterations, and muscle protein depletion combining to minimize tissue damage while enhancing the repair process (118). Another of these systemic responses to disease is an increase in the production by the liver of a number of plasma proteins which are known collectively as the acute-phase proteins (APP). Positive and negative acute phase proteins were found to be differentially expressed in the sample set. The changes in the concentrations of APPs are largely due to changes in their production by hepatocytes, but there has been increasing recognition of the importance of tissue localised extrahepatic APP changes. Positive APPs serve different physiological functions for the immune system; and Some act directly to destroy or inhibit growth of microbes. Positive APPs include: CRP, D-dimer protein, mannose-binding protein, alpha 1 antitrypsin, alpha 1 anti-chymotrypsin, alpha 2 macroglobulin, fibrinogen, prothrombin, plasminogen, complement factors, coagulation factors (e.g., F11), ferritin, complement and Serum Amyloid A proteins (118). In the recovery sample analysis, it is likely that the upregulation of negative acute phase APPs is due to their decrease in acute samples and relative return to normal (including apolipoproteins).

These acute phase proteins increase in the blood plasma in response to cytokine production that may be from several nonimmune and immune cells. In the case of DRESS, it is unclear if the main cytokine producers are effector T cells at the site of disease and organ level. The innate immune activation we see in the peripheral blood may thus just be a bystander of T-cell activation and tissue inflammation, as well as contribution from DAMPs released during tissue damage. De et al, has characterized innate

immune activation in the acute phase of DRESS, consistent with our findings (119). Interestingly, Tuberculosis-immune reconstitution inflammatory syndrome profiling in plasma is also characterized by an increased innate immune response and inflammatory signatures. TB-IRIS is an abnormal, excessive immune response against alive or dead Mycobacteria tuberculosis that may occur in HIV-infected individuals (120, 121). Specific biomarkers that have been identified for TB IRIS include immune activation markers, Immunoglobulins, and cytokines, in addition to APPs such as C-Reactive protein (122). These corroborate the findings present in this study which brings to the surface that there may be parallels that can be drawn between DRESS and TB IRIS from a proteomic, immunological standpoint.

No detailed proteomic analyses have been conducted in DRESS, yet *Cho et al* outlined soluble factors associated with DRESS including JAK/STAT signalling proteins, Fas/FasL, Granzyme B, Granulysin and TNF-α, IFN-γ (45). None of these or related proteins were identified, and this is likely due to the very low concentrations at which cytokines or other immune proteins are found in the serum (outside site of disease), and consequently, despite attempted depletion of high concentration plasma proteins these do not appear in listing of top differentially regulated proteins. This is a potential limitation of this thesis approach that will need consideration in future work.

Acute DRESS is characterised by TH2 infiltration of skin and organs e.g., liver, as well eosinophilia. There were multiple non-immune related proteins significantly upregulated ($p < 0.05$, $FC > 1.5$) in acute samples linked to actin-binding and thus cell structure, morphology, and functioning (including (ACTB) Actin, cytoplasmic 1, (ACTC1) Actin, alpha cardiac muscle 1, (VCL) Viculin). Actin assembles at the plasma membrane to provide rigidity to the cell, act as a scaffold to restrain membrane-bound proteins (for example, receptors) and enable endocytosis of receptors, such as Toll-like receptors (TLRs) (123). Microtubules and microtubule motors traffic cargo, such as autophagosomes, inside the cell. Cytoskeleton rearrangements caused by bacterial invasion or toxins are recognized by sensors which leads to the activation of innate immune signalling pathways. Its presence in the acute proteome may thus represent cell damage or relate to the considerable proliferation and infiltration of lymphocytes into affected DRESS organs (103, 124).

Two differentially regulated proteins that warrant special consideration between acute and recovery DRESS samples are (SELL) L-selection and (LRG1) Leucine-rich alpha-2-glycoprotein, both of which are

linked to TGFB (125). L-selectin mediates the adherence of lymphocytes to endothelial cells and promotes initial tethering and rolling of leukocytes in endothelia. LRG is synthesized in hepatocytes and its production is upregulated in response to systemic inflammation. Leucine-rich alpha-2-glycoprotein is involved in transforming growth factor beta signalling – a key counter-inflammatory and tissue repair pathway that may occurred even from early in DRESS pathogenesis (126). Alternatively, *Fujimoto et al* found serum LRG levels in TB patients to be significantly higher than those in healthy controls, indicating that TB disease burden may be driving this protein with the decrease during recovery related to increased TB treatment exposure and decrease mycobacterial loads which (126). The maintenance of immune surveillance and the generation of normal immune responses are dependent on leukocyte and lymphocyte migration to appropriate lymphoid and nonlymphoid tissues mediated by L-selectin. L-selectin-mediated lymphocyte recirculation is required for maintaining the appropriate tissue distribution of lymphocyte subpopulations including naïve and effector subsets such as regulatory T cells. L-selectin ligands can also be induced on the endothelium of inflamed tissues. Specifically, vascular L-selectin ligands are expressed at cutaneous sites of chronic inflammation (127). One study has recently shown that increasing L-selectin expression in cytotoxic CD8 T-cells is causal to viral clearance (103, 128).

Peptidyl-prolyl cis-trans isomerase A (p =, FC) and Selenop P ($p < 0.05$, FC >/= 1.5) were proteins to highlight. The former protein activates endothelial cells (ECs) in a proinflammatory manner by stimulating activation of NF-kappa-B and ERK, JNK and p38 MAP-kinases, and is involved in inhibiting the replication of viruses (129). It is understood that viral reactivation is a common factor contributing to DRESS pathogenesis, so Peptidyl-prolyl cis-trans isomerase A downregulation in the drug-positive and recovery samples, may reflect declining viral reactivation into the recovery phase. Selenop P protein was significantly downregulated in the acute samples. Selenop P protein is involved in the antioxidant pathway. More specifically, it is responsible for the extracellular antioxidant defense properties of selenium or might be involved in the transport of selenium (130). Oxidative stress, in the context of HIV, is interesting as HIV patients are more susceptible to oxidative stress (28). These individuals often have a Selenium deficiency which increases the risk of oxidative stress due to antioxidant proteins requiring Selenium to function (103). It is surprising to note that Selenop P is highly expressed in the patients during their drug reaction/follow up as this means that the oxidative stress is being reduced over the course of DRESS. Therefore, oxidative stress pathways may be relevant across DRESS disease .

Recovery analysis is characterized by a reduced acute phase response, innate immune response and cell mobility and adhesion

This study attempted to investigate and identify potential DRESS specific biomarkers that differentiated the recovery samples (i.e., early, and late recovery). This was an important analysis to be able to tease out immunoproteomic differences in patients that recover earlier than later. Upregulated proteins in the acute samples are similar in acute vs early recovery and acute vs late recovery analyses highlighting similar pathways are at play. Specific proteins to note include cell morphology, structure and adhesion proteins that were found in greater expression levels in acute samples versus recovery. (LGALS3BP) Galectin-3-binding protein was found to be upregulated in acute verses early recovery which not only functions in cell adhesion but also serves to activate host defences against viruses. This protein was not detected in the acute versus late recovery analysis. This may contribute to the clinical, phenotypic differences that occur between these patients including recovering at different time points.

Overall, recovery samples had a decreased acute phase response and increased plasma apolipoprotein oxidation (Apolipoprotein M and Apolipoprotein-IV). Plasma apolipoprotein oxidation is linked to immunosenescence in which there is a decline in immune efficacy and ability to maintain tolerance to self-antigens, resulting in increased vulnerability to infectious diseases (131). Oxidative stress compromises immune functions and increases cellular apoptosis. With recovery samples portraying immunosenescence, this brings to light that there is a suppression and/or abnormal immune specific functioning's during the course of disease. This finding may provide a link to the susceptibility and vulnerability these patients have to DRESS pathogenesis and possible co-infections.

Acute phase response evident during disease progression in patients 10001 and 10071

Patients 10001 and 10071 were identified as unique patients based off certain clinical details during their DRESS disease progression. The former, patient 10001, reacted to Rifampicin at 24 hours, but this was thought a transient flare reaction and the treatment was continued, only for the condition to worsen and necessitate stopping ~3 weeks later. The latter, patient 10071, had a drug challenge reaction to Pyrazinamide, but later tolerated this drug during her second episode of extrapulmonary

TB, with no initiation of anti-retroviral therapy in the intercurrent period. These patients also had multiple samples. Specific proteins that were identified to be differentially regulated were assessed in greater detail over the course of disease. One interesting finding to note was the acute phase reaction that took place with the new TB infection. This was evident by the increased expression of acute phase proteins including (SAA2) Serum Amyloid A-2, (CFP) Properdin, (FGL1) Fibrinogen-like protein 1 and (APOA4) Apolipoprotein IV proteins.

Oral drug rechallenge reactions in single drug reactors with predominantly delayed onset reactions, are characterised by strong acute phase responses and the differential upregulation of MPO and LYST

A key aim of this study was to further our understanding of the drug provocation reactions with the aim, and hope, of identifying potential biomarkers. These biomarkers could then be further validated and later used in clinical settings to provide quicker and more successful treatment options. In an attempt to investigate the molecular mechanisms at play during these drug provocation reactions, the acute samples were compared to the drug positive reactions. These two conditions clustered separately with multiple differentially regulated proteins contributing to this clustering.

Reaction 1 and 2 samples do have some proteins that function in similar pathways including the innate immune defence/response. This is understandable as both drug reactions still fall under DRESS disease and are thus immunologically associated. However, several plasma protein differences between reaction 1 and 2 samples were evident. Reaction 1 samples are heavily associated with lymphocyte mediated immunity (C2, C1QB, CRP and LYST) and have a stronger acute phase response (RBP4, TTR, CRP and C2). T-cell receptor (TCR)-mediated cross-recognition is a major mechanism in the pathogenesis of DRESS. The presence of T-lymphocytes is a universal clinical observation in most severe drug-allergy reactions (132). Certain skin symptoms in DRESS are considered to be correlated with cutaneous infiltration of cytotoxic CD8+ T-cells, whereas exanthemas seem to be mediated largely by drug-specific CD4+ T-cells. MPO and LYST are upregulated proteins in reaction 1 samples. (MPO) Myeloperoxidase is part of the host defense system of polymorphonuclear leukocytes and is responsible for microbicidal activity against a wide range of organisms. (LYST) Lysosomal-trafficking regulator is involved in the regulation of the number and size of cytotoxic T-cells, Natural Killer cells, macrophages, and dendritic cells. It is also involved in the production of pro-inflammatory cytokines (103). The increased expression of these proteins may indicate the strong activation across effector

immune cell types including T-cells, monocytes, and neutrophils, potentially absent from flare-up reactions which may be occurring in those reacting to multiple drugs

Reaction 2 samples were found to possess a weaker acute phase response (downregulated CRP, C2 expression) and innate immune response. The only noteworthy protein upregulated in reaction 2 samples is (LGALS3BP) Galectin-3-binding protein which functions in cell adhesion and host defense against viruses. The role of this is unclear, but it is interesting to consider if flare-up reactions in those multiple reactors relate to uncontrolled viral reactivation on exposures to pharmacological varied agents.

— Limitations to the study

The major limitation of this study related to limited sample size, particularly for HIV/TB co-infected probable/definite/possible DRESS, furthermore ensuring directly comparative sampling is also an impediment. Despite an ongoing prospective IMARI registry and biorepository there are many variables to align for sample inclusion: i) matched drug with phenotype, so FLTD DRESS as opposed to SJS/TEN or cotrimoxazole DRESS, ii) not all patients have positive drug challenges, and iii) the logistics of sampling during positive challenges is complex as reactions happen any time of day or night. Consequently, the sample timing of the patients posed another limitation, where differing timing may have meant difference in timing across DRESS evolution and TB burden/treatment dynamics, which increased clinical heterogeneity. Unfortunately, when working with clinical data, this variability is common and at times, cannot be prevented.

The small sample size also limited the ability to apply was the multiple testing correction during the data analysis and statistical stage. As only eight patients were used for this study, the comparisons were often done with very few samples and therefore, multiple testing corrections were unable to be done. In turn, this means that the results from the data collected and analysed can only be considered preliminary, only be used as a guide, and will require an enlarged sample size and further validation further testing and validation. This may be a reason behind certain correlations or pathways not being detected and molecular mechanisms remaining undetermined for some analyses.

The use of plasma as the study sample of choice also provided limitations, particular the dominance of high concentration/abundant proteins even with plasma depletion. For instance, the APPs and immunoglobulin chains may mask other more relevant proteins to DRESS disease pathogenesis and the differing immune pathways involved in distinct drug rechallenge reactions. DRESS predominantly involves skin and liver, and thus these site-of-disease samples may be more valuable to understanding differential immune pathways. These samples are difficult to attain especially during positive in vivo drug challenges but are being sought for future work.

— Future work

The small sample size of this work means that findings must be considered preliminary and expanded substantially further for robust conclusions. Proposed future studies include validating and confirming the differentially regulated proteins and pathways involved in the DRESS patients in mediating disease. There still remains a huge research gap in understanding the pathways in question and their link to DRESS pathogenesis. Further analysis would be valuable, especially to understand and validate the role of these highlighted proteins in the context of HIV-infection and disease progression itself, and in HIV infected patients who develop SCAR. As this proteomic work has not yet been performed before, future studies that use proteomic analysis on larger sample sizes to gain a deeper understanding on which proteins are highlighted as a result of SCAR, HIV/TB co-infection and treatment interruptions.

To better characterize and examine the phenotype at a cellular level samples, mass spectrometry on in vitro drug-stimulated peripheral blood mononuclear cells (PBMC) from the same patients will be performed. This will allow a complementary analysis with greater depth into non-secreted proteins. Studying PBMC's will provide a greater level of granularity of molecular mechanisms of drug positive reactions to characterize clinical differences on a proteomic level. Some of the patients in this study also have RNA-sequencing data from both peripheral blood PBMCs and skin biopsy samples that can be paired with this analysis to provide a more comprehensive look at DRESS related changes in the peripheral blood compartment.

— Conclusion

Although uncommon, SCARs remain a challenge in the clinical health setting. HIV infection is associated with increased risk of skin disorders including SCARs, and HIV-induced immune dysregulation is consistently thought to play a role in triggering onset of these conditions. Looking across the evolution of DRESS one notes innate immune stimulation early in the disease and also significant cellular stress and damage with excess cytoskeleton proteins. As the inflammation resolves, there is an increase in redox recovery and tissue repair. Lastly, proteomic characterization of single versus multiple drug reactors highlighted differential expression of proteins functioning in lymphocyte and leukocyte mediated immunity.

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**Reference for R Package:*

Kim-Anh Cao, Florian Rohart, Ignacio Gonzalez, Sebastien Dejean with key contributors Benoit Gautier, Francois. Bartolo, contributions from Pierre Monget, Jeff Coquery, FangZou Yao and Benoit Liquet. (2016). mixOmics: Omics. Data Integration Project R package version 6.1.1. https://CRAN.R-project.org/package=mixOmics

Supplementary Information:

Quality control figures:

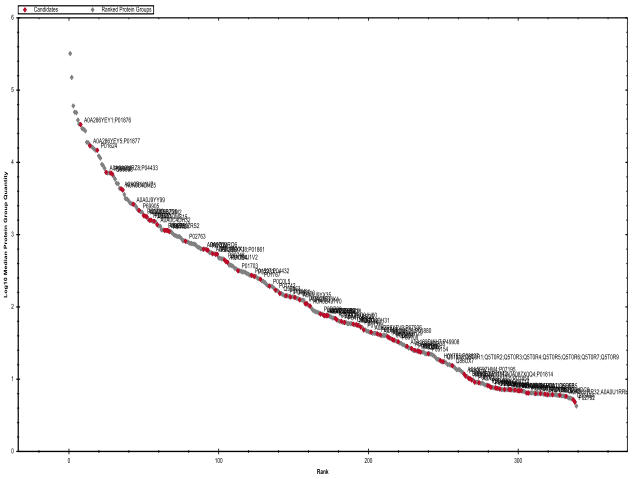

Figure S1: Ranked protein groups show the distribution of the different protein groups that were identified in the data as a function of how much of each protein was present in the sample on average *(i.e., how abundant were the proteins that were identified). In this case the proteins quantified were present across ~4.5 orders of magnitude*

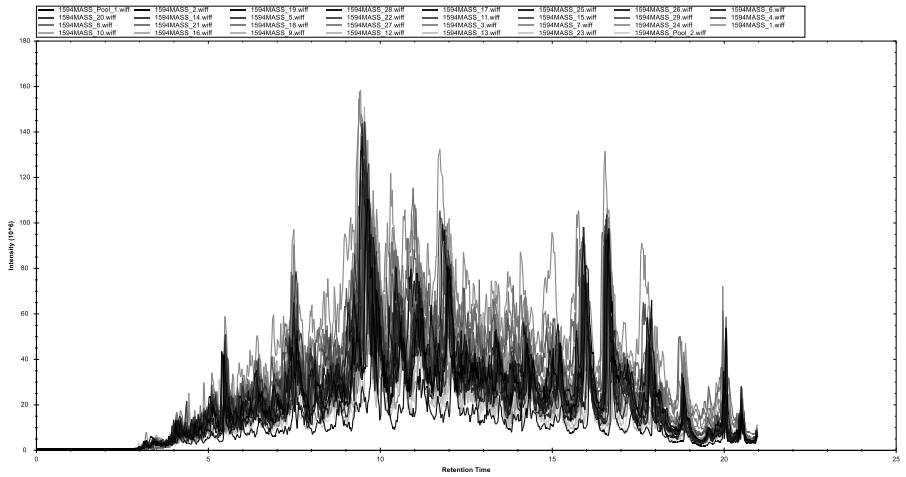

Figure S2: The TIC (total ion chromatograms) for each file show how consistently every sample ran. The produced data was very reproducible.

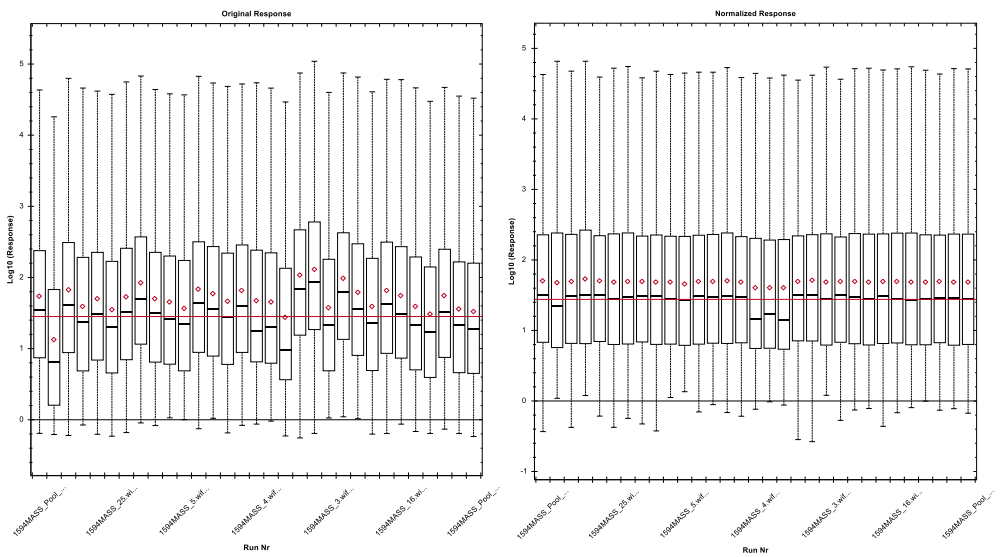

Figure S3: The data normalization shows you what the distribution of the intensities of the precursors looked like before and after normalization. *For a label free experiment, this is a means to determine that the loading was approximately equal per sample*

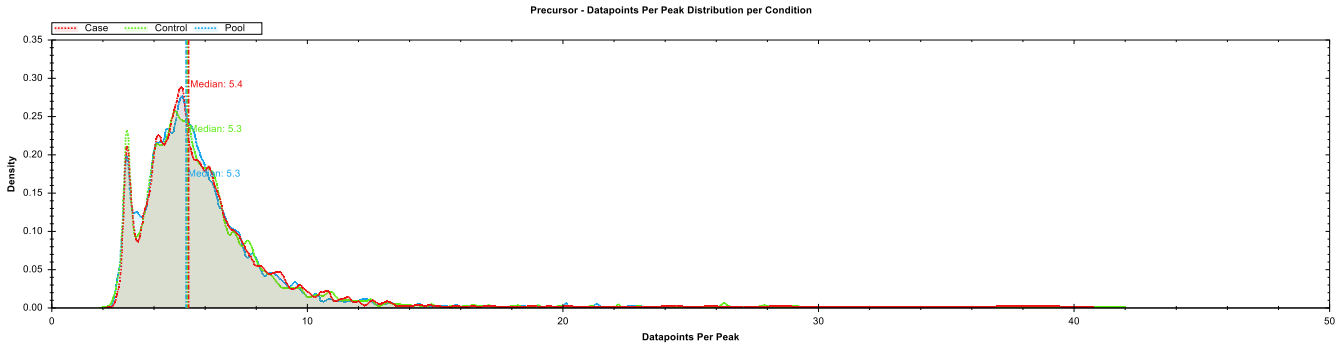

Figure S4: The data points per peak indicates the median number data points that were used to calculate the AUC for each precursor.

Analysis 3:

Heatmaps: (can be saved in order to zoom in)

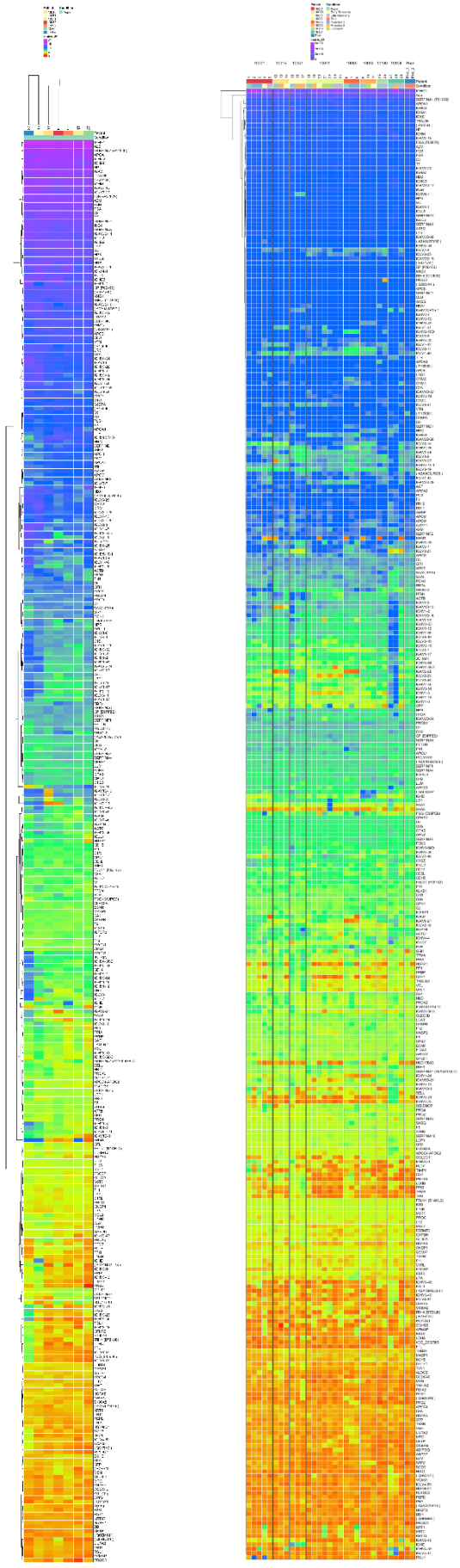

Figure S5 (left): A Heatmap showing the gene intensity level for all acute patients

Figure S6 (right): A heatmap showing the gene intensity level for all samples included in the patient cohort

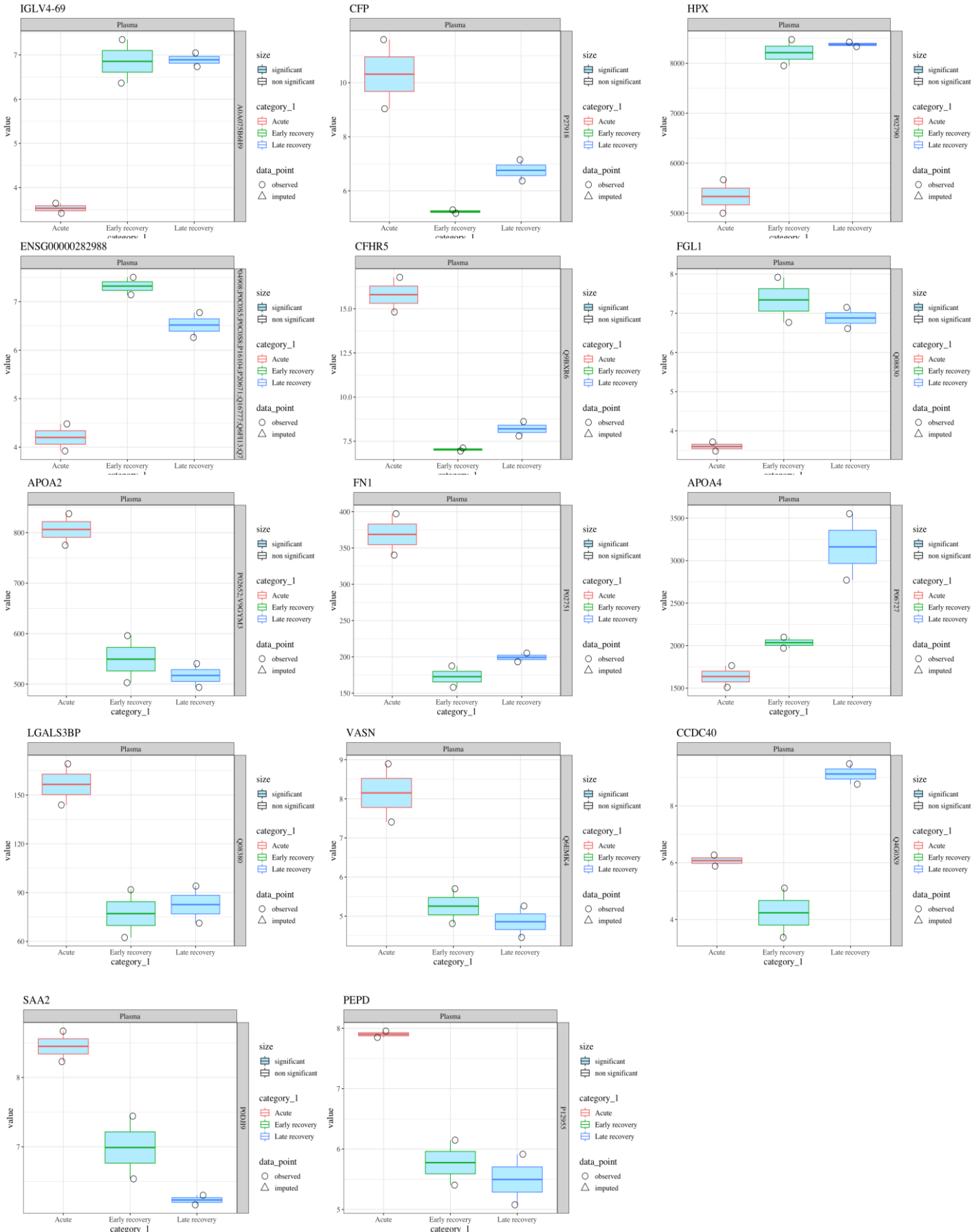

Figure S7: Box and Whiskers diagram which show the significantly up/downregulated proteins in the analysis